(51) International Patent Classification 5 :		(11) International Publication Number:	**WO 90/04650**
C12Q 1/68	**A1**	(43) International Publication Date:	3 May 1990 (03.05.90)

(21) International Application Number: PCT/GB89/01263

(22) International Filing Date: 20 October 1989 (20.10.89)

(30) Priority data:
8824592.3   20 October 1988 (20.10.88)   GB

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(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

(54) Title: A PURIFICATION PROCESS FOR COVALENTLY BOUND DNA/PROTEIN COMPLEXES

(57) Abstract

A process for separating covalent DNA/protein complexes from non-covalent DNA/protein complexes and unbound DNA comprising the steps of: (i) treating the DNA/protein complexes with a reactive derivative of polyethyleneglycol (PEG) and (ii) subjecting the production of step (i) to phase partition between an aqueous PEG solution phase and an aqueous phosphate solution phase.

A purification process for covalently bound DNA/protein complexes.

The present invention relates to processes for purifying and estimating covalent DNA/protein complexes and to materials produced by the process.

Current methods to estimate the formation of DNA/topoisomerase and other covalent DNA/protein complexes are time consuming and have other disadvantages. The alkaline elution technique of Zwelling, L.A. et al., Biochemistry, 20:6553-6563 (198 .) for detection of protein associated DNA breaks is very lengthy and difficult to standardise. It is also difficult to adapt this technique to recovery of DNA/protein complexes. The precipitation method of Trask, D.K. et al., EMBO J., 3:671-676 (1984) is also a multi-step procedure and although it can be used to recover complexes has the disadvantage that it is known to be most efficient for sections of DNA with many protein molecules in situ and very inefficient for single sites. This probably favours replication fork DNA, since topoisomerase II is very active here. Trask's method does precipitate predominately newly replicated DNA. Site-specific topoisomerase cleavage occurs as either clustered or single sites, and may therefore be under-represented in this method.

Phase systems of immiscible aqueous solutions of polymers and/or salts provide a means of separating cells and macromolecules on the basis of their differential affinities for the two component phases [Walter H, Brooks DE, & Fisher D. (eds.) Partitioning in aqueous two-phase systems, theory, methods, uses and applications in biotechnology. Academic Press, Orlando 1985].

It has now surprisingly been discovered that, by coupling polyethylene glycol (PEG) to the protein component of DNA/protein complexes, covalently bound DNA/protein complexes may be separated from non-covalently bound

DNA/protein complexes and unbound DNA using a suitable aqueous biphasic system comprising a top phase of PEG solution and a bottom, phosphate solution phase.

The present invention therefore provides a process for separating covalent DNA/protein complexes from non-covalent DNA/protein complexes and unbound DNA comprising the steps of:

(i) treating the DNA/protein complexes with a reactive derivative of PEG and

(ii) subjecting the product of step (i) to phase partition between an aqueous PEG solution phase and an aqueous phosphate solution phase.

Preferably covalently bound DNA/protein complexes are recovered from the aqueous PEG phase.

Without wishing to be bound by theory, it is believed that the PEG coupling conditions and/or the phase partitioning conditions result in dissociation of non-covalently bound DNA/protein complexes and, in view of the high affinity of DNA for phosphate solutions, [Albertson P-A. Partition of cell particles and macromolecules. 3rd Edition, John Wiley and Son, New York, 1986], it is only the PEG-linked covalent DNA/protein complexes which are drawn to the PEG phase.

Coupling of PEG to the DNA/protein complexes is preferably conducted by reacting the complexes with a reactive 2,2,2-trifluoroethanesulphonyl (tresyl) derivative of PEG, preferably tresyl monomethoxyPEG (TMPEG) which is described in our British Application No. 8824591.5.

The present invention will be further described with reference to the Figures of the accompanying drawings in which:

Fig. 1. shows schematically the coupling of PEG to DNA/protein complexes.

Fig. 2. is a graph showing the effect of sonication on the molecular weight of salmon sperm DNA, (12μ peak to peak amplitude, 20kHz150W).

Fig. 3. is a graph showing the effect of sonication on protein-free DNA partitioning.

Fig. 4. shows graphically the effect of extra rounds of phosphate extraction on recovery of DNA from the PEG phase.

Fig. 5 and Fig. 6 show the dose related increment in PEG phase DNA following treatment of cells with VP-16-213 (Fig 5) and UV (Fig.6).

Fig. 7, 8, 9, 16 and 21 shows scanning densitometer plots.

Fig. 10. is a graph of yields from PEG phase separation of Trask preparations.

Fig. 11. shows differential DNA recovery from retinoic acid induced and uninduced cells.

Figs. 12 and 13 shows results of differential hybridisation experiments.

Figs. 14 and 15 shows results of differential hybridisation experiments.

Figs. 17 and 18 shows the effect of retinoic acid and novobiocin on yield of DNA produced by the Trask method (Fig. 17) and the present method (Fig. 18).

Figs. 19 and 20 shows tubulin hybridisation to fractionated DNA from various induced or uninduced cells.

Figs. 22 and 23 show hybridisation signals from cloned DNA prepared by the process of the invention.

Fig. 24. shows increments in PEG phase DNA from induced or uninduced cells exposed to VP16.

Fig. 25. shows the effect of m-AMSA on DNA yield when partitioned according to the invention.

Fig. 26. shows the effect of VP16 on PEG-phase yield from cells pretreated with retinoic acid.

Fig. 27. shows the use of UV cross-linking to demonstrate retinoic acid induced DNA protein association.

The process of the invention exploits two phenomena (shown schematically in Figure 1). First, activation of PEG with tresyl chloride (panel 1, Figure 1) allows the PEG to be covalently attached to proteins (panel 2). This gives DNA/protein complexes very high affinity to the PEG-rich top phase, allowing the attached DNA to be recovered, while unattached DNA remains in the phosphate-rich bottom phase (panel 5). This can be used to recover or quantitate the protein-linked DNA. The level of DNA in the top (PEG) phase is dependent on the level of DNA/protein complexes in the sample, on the average size of DNA attached to protein (the latter is controlled for some applications by sonication, DNAse treatment, or restriction endonuclease digestion of the sample).

Secondly, the reaction mixture and/or phase partitioning conditions described here result in the non-covalently bound protein (with which the majority of eukaryotic DNA is associated) becoming detached from the DNA.

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The method therefore results in DNA that was unbound or non-covalently bound to protein being left in the phosphate phase. PEG is known to compact and alter the conformation of DNA. Although the precise nature of this change is still debated, we presume that detachment of non-covalently bound DNA occurs because of changes in the dimensions of the major and minor grooves of DNA (since these are critical for the non-covalent binding of proteins to DNA).

With the new partitioning method the presence of even single topoisomerase molecules will theorectically allow fractionation. The ability of a single PEG-modified topoisomerase molecule, or similar protein, to carry its attached DNA to the PEG phase, depends on the length of attached DNA, since it reflects the outcome of opposing forces (the DNA and PEG-protein compete to take the complex to the phosphate and PEG phases respectively). Successful fractionation of DNA attached to single topoisomerase molecules will therefore only be achieved if the attached DNA is short enough. This can be accomplished by sonication but we have also established the feasibility of using restriction enzyme of DNAse digestions while the topoisomerase II is still attached to the DNA. Shorter DNA fragments do, however have lower partitioning coefficients (Muller M Partitioning of nucleic acids. pp 227-266 in: Walter H, Brooks DE, & Fisher D. (eds.) Partitioning in aqueous two-phase systems, theory, methods, uses and applications in biotechnology, Academic Press, New York 1985) i.e., show a more equal distribution between the two phases, so additional rounds of extraction with the phosphate phase (or counter-current phase partitioning) will be required to compensate for this. We have shown that this is easily accomplished.

The present invention may be exploited in, for instance, the following:

1) Purification of DNA adjacent to classes of inducible genes (those where induction of down-regulation of transcriptional activity is accompanied by action of DNA topoisomerase I and/or II or other enzymes forming covalent intermediates with DNA). This provides a novel strategy for DNA cloning of inducible genes.

2) Purification of DNA at protein binding sites (for proteins not naturally covalently linked to DNA as they act, an additional step is required to attach the DNA to the protein after binding).

3) Assay of DNA topoisomerase activity.

4) Assay of DNA topoisomerase cleavage-site specificity.

5) Preparation of DNA protein binding sites using the "DNAse protection" principle.

6) Assays for DNA-protein cross linking agents .

7) Assays for inhibitors of DNA topoisomerase and other nicking/closing enzyme.

The invention further provides products comprising a DNA fragment covalently bound to a protein having at least one PEG moiety bound to the protein. Preferably the PEG moiety is a monomethoxy PEG group.

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THE INVENTION WILL NOW BE ILLUSTRATED IN THE
FOLLOWING EXAMPLES.

EXAMPLES 1 TO 11
a) ACTIVATION OF PEG WITH TRESYL CHLORIDE

To avoid hydrolysis of tresyl chloride all reagents
must be dried before use. Monomethoxy poly(ethylene)glycol
(MPEG) (Mr 5000, Union Carbide, USA) was dissolved in benzen
(B.P. 79-80°C) and the water-organic azeotrope (B.P. 65°C)
was distilled off. MPEG was recovered by removal of solvent
under reduced pressure and was finally dried by leaving
overnight at room temperature under vacuum. Dichloromethane
(AnalaR grade BDH, U.K.) was dried over molecular sieve A3
(crystalline potassium alumina silicate, BDH, U.K.) overnight
at room temperature using 100g per litre of solvent.

Dry MPEG (18g, 3.5mmol) was dissolved in dry
dichloromethane (45ml) at room temperature. The mixture was
cooled to 0°C, stirred magnetically and 1.125ml (14mmol) of
pyridine (AnalaR grade BDH,U.K.) and 1ml (9mmol) of tresyl
chloride (Fluka AG, Switzerland) at 0°C were added dropwise.
The reaction was allowed to continue at room temperature with
constant stirring for 1.5h before the dichloromethane was
removed by evaporating under reduced pressure. The white
solid was dried under vacuum overnight at room temperature.

This tresyl-PEG preparation (TMPEG) was washed to
remove pyridine before use. The TMPEG was dissolved in
methanol-HCl mixture (250:1) and allowed to precipitate -20°C
for 8 h. The resulting white solid was collected at 0°C and
the filtrate checked for pyridine content
spectrophotometrically (λ_{max} 255 nm). This procedure was
repeated by using methanol-HCl (1000:1) as washing mixture
until no pyridine could be detected. Finally, the pyridine-
free TMPEG (10-12g; 55-66% yield) was dried under vacuum for
several hours at room temperature. Sulphur content was 0.5%

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(theoretical content for 1 tresyl group per molecule of MPEG-
5000 is 0.62%). The TMPEG was stored desiccated at 4°C prior
to use.

b) PEG MODIFICATION OF DNA/PROTEIN COMPLEXES

TMPEG, 400mg/ml in 0.05M Na phosphate 0.125 NaCl
buffer pH7.5 was mixed with cell lysate or DNA/protein
complexes at a ratio 1:1 (v:v) on a rotating mixer for 2h at
room temperature. Since we do not know the number of lysine
molecules per molecule of topoisomerase II and there are
other proteins present, we chose to add TMPEG in excess.
Experiments with albumin indicate maximum partitioning to the
PEG phase with a TMPEG:lysine molar ratio 8:1 and
significantly increased partitioning was achieved at much
lower values. The ratios of DNA/protein complexes to TMPEG
used in these experiments therefore represent a gross excess
of TMPEG, but this may have additional advantageous effects
because of the effects of PEG concentration on DNA
conformation (4,5). Further experiments will be needed to
evaluate these two facets of the separation procedure
independently (using mixtures of TMPEG and PEG during the
covalent modification step).

Aliquots of TMPEG treated material were added
directly to the phase system. Where further enzymatic
treatments (e.g. with restriction enzymes) are planned it may
be advisable to neutralise unreacted TMPEG to prevent it
modifying these proteins. We have established, in our work
on immunoaffinity phase partitioning, that lysine or albumin
can be used to prevent further undesired reaction of the
TMPEG after the coupling step. In practice we found that
proteinase K is robust and, provided overnight incubations at
37°C are used, is little affected by the addition of TMPEG-
treated material. However, for all other procedures using a
second enzymatic procedure, 1M free base lysine (Sigma)

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dissolved in coupling buffer (1 vol) was added to the
reaction mixture (6 vols) and the mixture incubated at room
temperature for a further hour.

c) PHASE PARTITIONING

The phase system chosen consists of 10% (w/w)
poly(ethylene glycol) 6000 (BDH). 14% phosphate (ratio of
16.86g KH_2PO_4 and 40.20g $K_2HPO_4(3H_2O)$ and 76% distilled
deionised sterile water. The system was allowed to mix and
settle into an upper PEG-rich and a lower phosphate-rich
phase. These were separated and individually filtered
through 0.22um filter (Gelman Science Inc. Michigan)
aliquotted and stored at -20°C. This was done because it is
difficult to sample aliquots of the mixed phase system
without taking varied proportions of the two phases and this
would reduce the reproducibility of the method. For each
fractionation, the phase system was reconstructed usually
using the stated volume ratio of PEG:phosphate phases, with
no more than 15% of the total volume of the phase system
consisting of tresyl-PEG treated DNA/protein extract. For
experiments fractionating small DNA fragments the ratio of
PO_4:PEG phases was increased usually to 750:250ul and
multiple rounds of phosphate extraction were used. The
phases were mixed by vortexing and allowed to settle at 25°C
for 10 min. For multiple phosphate extractions (see above)
the PEG rich phase was then transferred to a fresh phosphate
phase and the procedure repeated (the number of rounds of
extraction is indicated with each experiment).

d) SONICATION OF DNA OR DNA/PROTEIN COMPLEXES
TO REDUCE THE AVRAGE LENGTH OF DNA

Pure salmon sperm DNA (used for the experiments in Example 1)
was diluted to 10 ug/ml in TE (10mM TrisCl, 1mM EDTA, pH7.4)
and 500ul aliquots were sonciated at 12u peak to peak, 20
KHz, 150 watts in an MSE bench sonicator on ice, taking care

to avoid frothing. For all other experiments, 500ul aliquots of tresyl-PEG treated HL60 cell DNA/protein lysate (containing protease inhibitors - see above) were sonicated for 5-60 seconds, using the same settings as above.

e) DNASe TREATMENT OF DNA/PROTEIN COMPLEXES TO PARTITION "PROTECTED DNA"

For the experiments of Example 6 DNA/protein complexes equivalent to 2.5ug DNA (prepared by the Trask method) were exposed to 10ug DNase I (bovine pancreatic DNAse, Sigma Ltd.) in 500ul of reaction mixture. The latter contained 50M Tris HCl; 25mM $MgCl_2$; 20mM Cl; 1mM $CaCl_2$; 10% w/v glycerol; 50ug/ml bovine serum albumin (BSA - Sigma Ltd.). After the exposure times indicated, the reaction was stopped using a final concentration of 1% SDS and 15mM EDTA.

Where DNAse was used as an extra step between two rounds of partitioning, recovered DNA/protein complexes in 160ul PEG-phase were exposed to 2.5ug of bovine pancreatic DNAse I in the presence of 65ul Tris EDTA pH 8.0, 12.5ul of 50mM MgCl and 12.5ul of 50mM $CaCl_2$ and 10ul of 10% BSA. Two minutes exposure was used

f) <u>RECOVERY OF DNA FROM THE PHASES AND QUANTITATION</u>

We evaluated several means of estimating the amount
of DNA in the phases. Many commonly used methods are not
applicable to the DNA while in the phases themselves.
Chemiluminescence prevented use of [H^3]DNA and scintillation
counting. The fluorescence of PEG prevented the use of
Hoechst 33258 (an intercalating dye used to estimate DNA by
fluorescence). PEG can be removed by chloroform extraction
and phosphate and other salts by an appropriate desalting
column.

The level of DNA in the PEG phase serves as a measure
of the DNA complexed to topoisomerase, but this cannot
readily be measured fluorimetrically in the presence of the
protein because of quenching. Phenol/chloroform extraction
is used to separate protein from DNA, but cannot be used
directly because protein covalently linked to DNA will cause
loss of the attached DNA (usually the DNA desired). The
topoisomerase must therefore be digested. Proteinase K
(Sigma Chemicals Poole) 20mg/ml aqueous solution was added
(100ul/ml of PEG or phosphate phase) and incubated at 37°C
overnight. This step can then be followed by an extraction
procedure to recover the DNA (e.g. by three rounds of
phenol/chloroform extraction using a 1:1 ratio (v/v)
(phenol/chloroform contained 0.1% 8-hydroxyquinolone - BDH
Chemicals Poole) and one round of chloroform/isoamyl alcohol
(24:1 V:V) to remove any remaining phenol. This procedure
effectively removes PEG but not phosphate. In order to
remove the latter (since ethanol precipitation of DNA is not
effective if salt concentrations are too high) 110ul of the
extracts from both PEG-rich and phosphate-rich phases were
loaded onto 1.2ml columns of Sephadex G50 (Pharmacia) packed
in a 1ml syringe. The Sephadex was pre-equilibrated with
10mM Tris HCl, 100mM NaCl 1mM EDTA pH 8.0 (STE). Columns
were spun at 400g for 4 min. DNA was recovered from the

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eluate by precipitation in 2 volumes of absolute alcohol at
-70°C for 1 hour followed by centrifugation at 11,000g for 10
min. in a microfuge (MSE instruments) and the pelleted DNA
resuspended in 20ul of Tris EDTA: 10mM Tris:HCl, 1mM EDTA
pH7.4 (TE). This DNA can then be estimated by conventional
means. For the examples above it was either estimated by
ultraviolet absorbance spectrophotometry, or, where we wished
to examine the size distribution of the loaded and recovered
DNA (since partitioning behaviour varies with DNA fragment
size), we used agarose gel electrophoresis. A 6ul aliquot of
each sample was run in a 1% (w/w) agarose gel (100x75mm) with
ethidium bromide 1ug/ml in Tris running buffer: Tris borate
0.089M, boric acid 0.089M, 0.002M EDTA (TBE) at 24 volts (2.4
V/cm) for 1 hour. The gel was photographed on a UV
transilluminator at 245nm with Polaroid 667 film. The DNA
was estimated from the integrals of scanning densitometer
plots of the negatives (Joyce Lobel Instruments Gateshead) by
comparison with know amounts of DNA. Experiments adding
known amounts of DNA individually to PEG and phosphate phases
with this procedure showed that recoveries vary for the two
phases. For a rapid quantitation samples were spotted onto
an agarose slab, photographed on a transilluminator as above
and compared to a set of DNA standards.

Losses are significantly lower for the PEG than for
the PO_4 phases (see Example 1). Thus to estimates the "true"
partitioning behaviour of samples, results have to be
corrected for this differential loss and recovery standards
were included for this purpose in specified experiments.

When calculating recoveries with respect to loaded
DNA to estimate the DNA lost in the procedure, in order to
exclude losses for subsequent steps (PK digestion
phenol/chloroform extraction ethanol precipitation etc.) an
aliquot identical to the DNA loaded was treated as the
recovered samples and used a a reference against which to

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calculate the % loaded DNA recovered.

Preparation of HL60 cell protein-associated DNA

     HL60 cells in log phase growth were harvested by
centrifugation at 400g for 6 min. and resuspended in serum
free medium (RPMI Gibco Ltd.). at 5×10^6cells/ml. Cells
were lysed in the presence of protease inhibitors by the
addition of 10% aqueous SDS to a final concentration of 1%
with 1% v/v Triton X-100 (Sigma), 15mM EDTA and 1mM
phenylmethylsulphonylfluoride (Sigma) from a stock solution
of 100mM in methanol. The cell lysate was vortexed
aliquotted and stored at -20°C.

     In some experiments cells were pre-treated for 70 min
with 10^{-6}M all trans retinoic acid (sigma) or VP16-213 10^{-5}M
(Vepesid, Bristol Meyers U.K.) for the times stated. The
timing of retinoic acid treatment was selected because
previous studies indicate that protein associated DNA breaks
induced by differentiation are present after this time
interval. Controls were sham treated with the relevant
diluent for the same times. Appropriate sham treatment of
controls is particularly important in some types of
experiment (e.g. UV protein crosslinking) where cell
manipulations clearly influence DNA/protein associations and
hence yields. For such experiments, individual results must
be expressed with respect to matched sham treated controls
rather than, say, to a single "untreated" or time zero
controls.

SDS/KCl precipitation of DNA/protein complexes (Trask
procedure)

     This method is essentially that given in the
literature (loc. cit.) except that calf thymus DNA was
omitted from the binding reaction buffer and vigorous
vortexing was not used (so that long DNA fragments were not
sheared).

g) INCREASING DNA-TOPOISOMERASE CROSSLINKING WITH VP16

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Log phase HL60 cells, grown in RPMI 1640 supplemented with
10% foetal calf serum (both Gibco - Paisley Scotland), were
harvested and resuspended to 6.5×10^6/ml. The cells were then
allowed to re-equilibrate in the incubator at 37°C.
Etoposide (VP 16-213 Bristol Meyers, NY state USA) was
serially diluted in serum free RPMI with an appropriate
diluent control, to final concentrations of 10^{-5}M down to
10^{-9}M and an equal concentration of diluent throught the
gradient. This was then allowed to react with the cells for
15 mins at 37°C. The reaction was finally stopped by the
addition of sodium dodecyl sulphate and Triton X-100 (both
Sigma Chemicals - Poole England) to a final concentration of
1% (v/v). Phenyl methyl sulphonyl fluoride (PMSF) and
disodium ethylenediaminotetraacetic acid (EDTA both Sigma
Chemicals - Poole England) were also added at a final
concentration of 1mM and 15mM respectively.

Tresyl monomethoxy poly(ethylene)glycol (TMPEG) was dissolved
in coupling buffer (0.5M NaPO4 0.08M NaCl pH 7.5) to a
concentration of 400mg/ml and an equal ratio (200ul: 200ul)
was allowed to react with each of the cell lysates on a
rotating turntable at room temperature for two hours.

Six 1000ul phases were constructured (500:500ul) from PEG and
Phosphate and 100ul of the TMPEG:cell lysate mixture was
added to each phase. These were then vortexed and allowed to
settle at 25°C for 10 mins. The phases were then separated
and incubated at 37°C overnight with proteinase K (Sigma
Chemicals - Poole England) at a final concentration of
100ug/ml. The phases were then cleared of protein by
extraction using two rounds of phenol/chloroform with 0.5%
8-Hydroxyquinoline (all BDH Chemicals - Poole England).

An equal aliquot (10ul) of ev ry sample was run for one hour

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at 25v/cm through a 1% agarose gel with Tris borate running buffer (0.089M Tris borate, 0.089M boric acid, 0.002M EDTA (pH 8.0) all BDH chemicals - Poole England). The gel was then photographed under ultra violet light of wavelength 254nm using Polaroid 667 instant film. The negatives were canned with a Joyce Lobel densitometer and the resultant integral's were used to calculate the DNA recovered with respect to the untreated control.

h) <u>UV CROSSLINKING OF NON-COVALENTLY BOUND PROTEINS TO DNA</u>
   Reference is Gilmour and Lis PNAS 81 p4275-4279;1984

Log phase HL60 cells growing in RPMI1640 medium supplemented with 10% foetal calf serum (both Gibco) were taken and spun at 400g for 4 minutes. These cells were then resuspended to the required concentration. A 1ml aliquot was then placed in a small petri dish (Nunc products) and exposed to UV light at a wavelength of 254 nm and intensity 5W/cm2 at 12.5cm. Cells were then collected by centrifugation in an Eppendorf tube at 6500g for 5 minutes. The cells were resuspended in 200 microlitres 10 mM Tris HCl pH 7.4 1mM EDTA (T.E.) sodium dodecyl sulphate, SDS (Sigma) and Triton X - 100 (BDH) to a final concentration of 1% and disodium EDTA (Sigma) was added to a final concentration of 15 mM. The samples were then cooled to 4°C and sonicated with an MSE bench sonicator on ice for 30 seconds, 12 micrometers amplitude peak to peak, 150 Watts and 20 kHz. This reduces the modal size of the DNA to about 800 basepairs. The negative control sample was incubated at 56°C for 1.5 hours with proteinase K (Sigma) to a final concentration of 5 micrograms per microlitre. This sample was then treated as the others.

Tresyl methoxypoly(ethylene glycol) TMPEG was prepared to 400 mailgrams per millilitre buffer in 0.05 sodium phosphate 0.125 sodium chloride pH 7.5 and was mixed

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in a ratio of 1:1 (v/v) with the U/V irradiated and
unirradiated samples for 2 hours at room temperature.

After incubation with TMPEG all the material was
added to a phase system composed of 750 microlitres
phosphate-rich and 250 microlitres PEG-rich phases. The
system was constructed using 14.7 % w/w poly (ethylene
glycol) 6000 (BDH), 11.2% w/w phosphate (ratio of 16.86 grams
KH2PO4 and 40.20g K2HPO4 (3H2o) both BDH) and 74.1% distilled
deionised sterile water. The phases were vortexed and
allowed to settle for 10 minutes at 25°C after which the
upper PEG-rich phases were removed to fresh 750 microlitre
phosphate rich bottom phases. This was then repeated to
leave one top phase and four bottom phases per condition.

Each condition was then incubated at 37'C O/N with
proteinase K to a final concentration of 5 micrograms per
microlitre. The phases were then extracted with two equal
volume rounds of phenol-chloroform (phenol:chloroform 1:1 v/v
containing 0.1% hydroxyquinoline, all BDH) and a final
extraction by chloroform-isoamyl alcohol 24:1 (both BDH) to
remove any residual phenol. This removes any PEG present but
not any phosphate. The phosphate must be removed as Ethanol
precipitation of DNA is ineffective in high salt
concentrations. The phosphate is removed spinning a sample
down a 1 ml syringe packed with Sephadex G-50 (Pharmacia)
which has been equilibrated with STE pH8 (10mM Tris HCl pH8;
1mM EDTA;100mM NaCl). The eluate is then precipitated with
two volumes of cold absolute alcohol at -70'C overnight.

The DNA was then spun for 10 minutes at 11,000 r.p.m.
in a MSE microcentrifuge and resuspended in 10 microlitres
T.E. pH 7.4. This was then run on a 1% agarose gel for 2
hours at 2.5 volts per centimetre. The gel was photographed
on a UV trans-illuminator with Polaroid 665 film. The
negatives scanned with a densitometer (Joyce Lobel) and the
integrals were used to calculate the amount of DNA present

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using a known salmon sperm standard.

The results were plotted as DNA present in the top phase as a percent of the starting amount and also as the amount in micrograms recovered from the top phases.

i) <u>PRE-EXPOSURE OF CELLS TO NOVOBIOCIN TO INHIBIT DNA
TOPOISOMERASE II AND HENCE REDUCE DNA ATTACHMENT</u>

HL60 cells were pre-incubated with 10^{-3}M novobiocin (Sigma Ltd.) for 60 min at 37°C under standard culture conditions (see above) and were then either exposed to retinoic acid (10^{-6}M for 70 min) or sham treated with diluent. Toxicity tests showed that less than 5% cells died with this dose of novobiocin when assessed by nigrosin. Novobiocin is an established inhibitor of DNA topoisomerase II that acts in a different manner to the epipodophyllotoxins and intercalators, in that, unlike the latter it does not stabilise cleavable complexes of enzyme and DNA, but tends to inhibit binding of the enzyme to DNA. However it was recently shown that the agent has a complex interaction with the enzyme in that with alterations in the ratio of drug:enzyme both inhibitory and stimulatory effects can be observed (Collins and Johnson Nucl Acids Res 7:1331;1979)

j) <u>DIFFERENTIAL HYBRIDISATION</u>

The hybridisation strategy using PEG and phosphate phase DNA from matched differentiation induced and uninduced cells is explained in Example 7.

The filters were prepared by the dot blot method of FC Kafatos, CW Jones and A. Efstratiadis. Nuclec Acid Research 7:1541;1979. Lifted from The Guide to Molecular Cloning Techniques, edited by S.L. Berger and A.R. Kimmel Academic Press 1987.

1) Preparation of nitocellulose or nylon GeneScreen Plus
filters (New England Nuclear Research Products - Boston USA)
with DNA from DNA/protein complexes recovered from cells
induced by all trans retinoic acid or phorbol-12-myristate
13-acetate (PMA) (both Sigma Chemicals - Poole England).

Usually 4 dilution of DNA were added to the filter with
0.05 ug as the highest dilution and three subsequent dots
with doubling dilution. Two to three replicate filters were
made with independent loading denaturing etc. Samples were
dilutes with T.E. pH 8.0 (10mM Tris.Cl 1mM
Ethylenediaminetetraacetic acid, EDTA) to 0.1ug of DNA. Each
sample then had addition of TE pH 8 to bring the volume to a
standard 50ul. All the samples were heated to 70°C with 0.1
volume of 3M sodium hydroxide (BDH Chemicals - Poole England)
for 45 minutes. This denatured the DNA and destroyed any
RNA, if present. The samples were then allowed to cool to
room temperature and 55ul 2M ammonium acetate (BDH Chemicals
- Poole England) was added. Serial dilutions were then
carried in 1M ammonium acetate. The DNA was loaded onto the
nylon (or nitro cellulose) with a 'home made' Dot - blot
device and allowed to stand at room temperature for at least
30 minutes prior to being sucked through by vacuum. Filters
were then dried between 3MM paper (Whatman Paper - Maidstone
England) and then baked for two hours at 80°C under a vacuum
(optional for nylon filters). The filters were then pre-
hybridised, using 6x SSC, (0.9M Saline, 0.9M Sodium Citrate
pH 7.0 Both BDH Chemicals - Poole England), 0.5% SDS, (Sodium
Dodecyl Sulphate, Sigma Chemicals - Poole England) 5x
Denharts olution (0.5g Picol, 0.5g polyvinylpyrrolidone and
0.5g Bovine Serum Albumen Pentax Fraction V - all Sigma
Chemicals, Poole England) and 100mcg/ml of highly sonicated
salmon sperm DNA (Sigma Chemicals - Poole England) in 200mls
of distilled deionized water. The filters were prehydridised

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for 3.5 hours at 68°C, with agitation, in the shaking waterbath. After pre-hybridisation, the fluid was poured off and hybridisation fluid was added which consisted of 6x SSC, 0.5% SDS, Denharts, 100 mcg/ml denatured salmon sperm DNA and 0.1M EDTA in 100ml volume. To this was added a probe radio labelled with $dCT^{32}p$, using the random primer method kit (Amersham Plc - Amersham England) and the amount of incorporation was assessed using adsorption to Whatman DE-81 filter paper Manniatis loc.cit. with washing to remove free nucleotides. Hybridisation was allowed to proceed again at 68°C with agitation for about 17 hours. Post hybridisational washes were twice 45 minutes and then twice 30 minutes of 1x SSC, 1x Denharts and 0.1% SDS in 100ml distilled deionized water which had been pre warmed to 68°C. This solution was added to the filters after the hybridisation fluid had been removed. The post hybridisational washes were also carried out with agitation in the waterbath. These washes were then followed by two final 20 minute rinse washes also with agitation in the waterbath with 100ml of pre warmed 0.1x SSC at 68°C. The filters were then blotted between sheets of Whatman 3MM paper and then allowed to dry thoroughly in air. The filters had a count of no more than 2-5 cpm above background and were exposed to Fuji RX 100 medical X - ray film (Fuji Photo Film Co. Ltd. - Japan) with two rare earth intensifying screens (Du Pont Cronex Quanta III, Du Pont Industries U.K.) at -70°C for 24-48 hours depending upon the result obtained.

The probes used included c-myc a 8Kbp ECo R1/Hind III fragment of genomic DNA (Dalla-Faura et al PNAS. 79:6497;1982.) as well as a 1.5Kbp cDNA fragment of c-fos (truncated with respect to the 5' end, lacking the 1st exon) (Murphy and Norton personal communication) as well as a retinoic acid plus (R+) probe which was prepared as follows. Retin ic acid induced cells were used to produc protein

associated DNA, prepared by the method of Trask et al. This
material (5.0ug) was then exposed to 0.05 ug Bovine
Pancreatic DNase 1 (Sigma Chemicals - Poole England) for a
period of five minutes in the presence of 50mM Tris.Cl pH
8.0, 10 % v/v Glycerol, 0.1% Bovine Serum Albumen, 250mM Mg^{2+}
10mM Ca^{2+} and 200mM K^+ (all Sigma Chemicals Poole - England)
in 250ul volume. A similar probe (R+) was prepared form
analogous, undifferentited HL60 cells.

The reaction was stopped by bringing the reaction
mixture up to 1% SDS and 15mM EDTA. An equal volume of
400mg/ml TMPEG (tresyl monomethoxy polyethylene glycol) was
allowed to react to the protein present for two hours at room
temperature and then was partitioned on a 250/270 ul
PEG:PO_4phase system with five rounds of PO_4. The DNA was
then liberated from the protein by the addition of proteinase
K (Sigma Chemicals - Poole England) to 20mg/ml overnight at
37°C. The samples were then extracted with two rounds of
phenol/chloroform followed by a single extraction with
isoamyl alcohol/chloroform.

k) Cloning of topoisomerase-associated DNA prepared by the
   new procedure

Two different cloning techniques were evaluated, but
in principal any standard technique could be used.

a) The homopolymer tailing technique was used to
introduce the prepared DNA fragments into dCTP tailed plasmid
(puc8). This method was selected because no differential
handling of topoisomerase cleavage sites, sheared ends or
restriction sites is involved (we wanted to avoid differences
in efficiency of processing fragments with and without the
topoisomerase staggered cleavage sites). Transformation
followed by growth of plasmid in and recovery from DH5 E.
coli, demonstrated that recombinant plasmids were produced.
However the fficiency of this method was relatively low.

b) The second strategy for the cloning of
topoisomerase-associated DNA was as follows: the overhang
created by topoisomerase II cleavage is endfilled with Klenow
(Amersham U.K.) and then Sa I linkers (Pharmacia) ligated to
the blunt ends. The DNA is then digested with Eco RI and Sal
I (Eco RI to digest the largest DNA fragments down to a
reasonable size and Sal I to create sticky ends). The DNA is
then ligated to pUC 18 which has been digested with Eco RI
and Sal I and transformed into E.coli DH5 cells (BRL). This
procedure should clone both randomly cleaved ends of DNA as
well as the topoisomerase associated DNA, but will tend to
exclude Sal I/Sal I fragments cut from sections of DNA
distant from the topoisomerase II cleaage site.

Using Xgal plates, for some unknown reason this material
gives many blue colonies which are produced on
transformation in addition white colonies. This was despite
having ligated vector alone and seeing that it was very
inefficient at producing clones (as expected). The
efficiency of producing white colonies varied between batches
of ligated vector/inserts. It is possible these blue
colonies contain very small inserts and a sample should
therefore be examined before these are discarded.

The DNA fractionated by the new method initially proved
resistant to digestion with restriction enzymes however this
was over come by first passing the DNA through QIAGEN
(Trademark) tips. The DNA for cloning by this procedure
should be kept as large as possible to reduce the number of
randomly cleaved ends available for cloning.

Cloning may be impoved by using vector cut with Sal I alone
and then treated with calf intestinal phosphatase to remove
terminal phosphate groups. If there is a Sal I site between

the artificially created Sal I site at the topoisomerase
cleavage site by the attachment of a Sal I linker and the
nearest Eco RI site, then these will be cloned (as will as
other Sal I-Sal I fragments).

Cloning of DNA (cf. Maniatis et al, Molecular Cloning : A
Laboratory Manual, Cold Spring Harbor, 1982

1) preparation of DNA for cloning

QIAGEN Tip Purification of fractionated DNA

Dilute sample with water to 130 ul

Add 70 ul 1.5 M NaCl/250mM MPS pH7.0

Equilibrate QIAGEN tip 5 with 300 ul buffer A

Absorb DNA on to tip by pippetting through tip 4 times

Wash off linkers with 5x750 ul buffer C

Elute DNA in 3x200 ul buffer F

Add 0.48 ml (0.8 vol) isopropanol and place on ice 15 min

Microfuge 30 min

Wash pellet with 70% ethanol

Microfuge 30 min

Dry pellet under vacuum

Dissolve in TE pH 7.5

Blunt Ending of DNA

2 ul	10xrepair buffer
5 ul	1mM dNTPs (1 mM each)
6 ul	TE pH 7.5
5 ul	DNA (2.0 ug)
1 ul	Klenow (5U/ul

Incubate 1 hour at room temperature

Heat at 65°C for 5 min (to heat inactivate enzyme)

Phosphorylation of Linkers

1ul	10xkinase buffer
1 ul	linkers (1 ug)

1 ul   T4 poynucleotide kinase

7 ul   ddw (double distilled water)

Incubate 1 hour at 37°C

Ligation of DNA to Linkers

20 ul   blunt ended DNA

6 ul   5xligase buffer (BRL)

5 ul   kinased linkers

10 ul   ddw

5 ul   T4 DNA ligase (1 U/ul)

Incubate at 4-15°C overnight

Add 50 ul phenol/chloroform and mix

Microfuge briefly and reextract with phenol/chloroform

Extract with chloroform

Back extract with 50 ul TE pH 7.5

Add 4 ul 5 M NaCl to 100 ul DNA

     400 ul 100% cold ethanol

Chill at -70°C then microfuge 15 min

Vacuum dry pellet

Restriction Endonuclease Digestion

40 ul   ddw (to dissolve DNA)

5 ul   10xbuffer (BRL H buffer for Eco RI and Sal I)

1 ul   Eco Rl (90 U/ul)

Incubate at 37°C for 1 hour

Heat inactivate at 68°C for 5 min

Add 4 ul Sal I (10 U/ul)

Incubate at 37°C for 4 hours

QIAGEN Tip Removal of Linkers

Add to 50 ul of DNA

        100 ul ddw

       25 ul 5 M NaCl

       7.5 ul 1 M MOPS pH 7.0

Equilibrate QIAGEN tip 5 with 300 ul buffer A

Adsorb DNA on to tip by pippetting through tip 4 times

Wash off linkers with 5x750 ul buffer C

Elute DNA in 3x200 ul buffer F

Add 0.48 ml (0.8 vol) isopropanol and place on ice 15 min

Microfuge 30 min

Wash pellet with 70% ethanol

Microfuge 30 min

Dry pellet under vacuum

Dissolve in 40 ul of ddw

Run 2 ul in a 0.8% agarose gel to determine average size

2) Preparation of vector for cloning

Preparation of pUC 18

16 ul   pUC 18 (0.25 ug/ul ie 4 ug)

2 ul   10xbuffer (BRL H buffer for Eco RI and Sal I)

1 ul   Eco RI (90 U/ul)

Incubate at 37°C for several hours

Run 1 ul on a 1% minigel to ensure cut to completion

Add 1 ul Sal I (10 U/ul)

Digest at 37°C for several hours

Add 110 ul ddw, 70 ul 1.5 M NaCl,120 mM MOPS pH 7.0

Remove small inserts using QIAGEN tips as above

Dissolve in ddw to a concentration of 0.1 ug/ul

Dephosphorylation of Vector

5 ul   10xCIP buffer

45 ul   vector (2 ug)

1 ul   calf intestinal phosphatase

Incubate at 37°C for 30 min

Add 1 ul CIP and incubate a further 30 min

Add   40 ul   ddw

        10 ul   STE (saline Tris EDTA)

        5 ul   10%SDS

Heat at 65°C for 15 min

Add 4 ul 5 M NaCl, 200ul cold ethanol and precipitate DNA at
-20°C

Microfuge for 15 min, remove supernatant and dry under vacuum

Dissolve in ddw to a concentration of 0.1 ug/ml

3) Ligation of vector to insert DNA

0.1 ug pUC 18 (cut with Eco RI/Sal I)

Insert DNA (at ratio 5:1 (mol:mol) and others)

ddw to 20 ul

Heat at 68°C for 5 min

Cool on ice

Add   4 ul 5xligation buffer

        1 ul T4 DNA ligase

Incubate at 15°C overnight

4) Transformation of E. coli (DH5 competent cells)

As per BRL protocol

Thaw cells on ice

Dispense into 20 ul aliquots

Add 1 ul of vector/insert DNA (diluted 1:5 in TE)

Incubate on ice for 30 min

Incubate at 42°C for 40 sec then place on ice

Add 80 ul SOC and incubate at 37°C for 60 min with shaking
(225 rpm)

plate onto X-gal plates and incubate overnight

Pick colonies into 100 ul L Broth + ampicillin and grow
overnight

Add 100 ul 30% glycerol and store at -70°C

5) Solutions and buffers

TE

1 mM EDTA

10 mM Tris

10xRepair buffer

0.5 M Tris pH 7.4

70 mM $MgCl_2$

10 mM dithiothreitol

10xKinase buffer

0.66 M Tris pH 7.6

10 mM ATP

10 mM spermidine

10 mM $MgCl_2$

150 mM dithiothreitol

2 mg/ml BSA (molecular biology grade)

10xCIP buffer

0.5 M Tris pH 9.0

10 mM $MgCl_2$

1 mM $ZnCl_2$

10 mM spermindine

6) Church hybridization

Reference

Church, G.M., and Gilbert, W. (1984) Genomic Sequencing,
Proc. Natl. Acad. Sci. USA 81, 1991-1995

Solutions

1.0 M Soldium Phosphate Buffer 7.2

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257.0 g $Na_2HPO_4.12H_2O$
56.8 g $NaH_2PO_4.2H_2O$
Make to 1000 ml with ddw
(May require heating to dissolve)

or

134 g $NaH_2PO_47H_2O$
4 ml   phosphoric acid
Make to 1000 ml with ddw
(May require heating to dissolve)

Church Prehybridization Buffer

0.5 M NaPi pH 7.2

7% SDS

1 mM EDTA

Stock	200 ml	500 ml	1000 ml
1 M NaPi pH 7.2	100 ml	250 ml	500 ml
20% SDS	70 ml	175 ml	350 ml
0.5 M EDTA	0.4 ml	1.0 ml	2.0 ml
ddw	29.6 ml	74 ml	148 ml

Church Washing Solution

40 mM NaPi Ph 7.2

1% SDS

Stock	1000 ml	2000 ml	5000 ml
1 M NaPi pH 7.2	40 ml	80 ml	200 ml
20% SDS	50 ml	100 ml	250 ml
ddw	910 ml	1820 ml	4450 ml

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<u>Procedure</u>

Prehybridize filter in 50-100 1/cm^2 at 65 C in plastic bag 2-6 hours

Add probe and hybridize overnight (16-24 h) at 65 C

Wash 2x5 min in Church wash at room temperature

Wash 2x30 min in Church wash at 65 C

Autoradiograph overnight with intensifying screen

7) Media, plates ad reagents

<u>SOC</u>

2g   bactotryptone

0.5 g   yeast extract

1 ml   1 M NaCl

0.25 ml 1 M KCl

Autoclave in 97 ml ddw

Cool to room temperature

Add 1 ml 2 M Mg^{++} and 1 ml 2 M glucose

Make to 100 ml

Filter sterilize

<u>L Broth</u>

10 g   Bactotryptone

5 g   Bactoyeast Extract

10 g   NaCl

Made to 1000 ml with ddw

Autoclave to sterilize

Cool then add 1 ml 25 mg/ml ampicillin

<u>L Agar</u>

1.5 g bacto agar in 100 ml L Broth

Auto clave to sterilize

Cool then add 1 ml 25 mg/ml ampicillin and pour plates

<u>X-gal</u>
20 mg/ml in dimethylformamide
100 mg x-gal in 5 ml dimethylformamide
X-gal: 5-bromo-4-chloro-3indoly- -D-galactopyranoside
Store at 4 C

<u>O.1 M IPTG</u>
119 mg IPTG in 5 ml sterile ddw
Filter sterilize, then aliquot into microfuge tubes
Store at -20 C
IPTG: isopropyl-thiogalactopyranoside

<u>25 mg/ml Ampicillin</u>
250 mg ampicillin dissolved in 10 ml ddw
Filter sterilize, then aliquot into microfuge tubes
Store at -20 C

<u>X-gal Plates</u>
Cool 100 ml L agar to < 50
Add   400 ul ampicillin at 25 mg/ml
               300 ul X-gal at 20 mg/ml
               100 ul at 0.1 M
Pour plates. Store at 4 C wrapped in cling film

EXAMPLE 1

THE PEG-PROTEIN COUPLING PROCEDURE INCREASES THE AFFINITY OF
PROTEIN-BOUND DNA FOR THE PEG PHASE AND ALLOWS RECOVERY AND
QUANTITATION OF THAT DNA

Before attempting to demonstrate the extent to which
PEG-phase recovery is due to the coupling of PEG to the
protein attached to the DNA, it is important to appreciate
that PEG-phase recoveries of free DNA are highly influenced
by DNA fragment length. This therefore needs to be
considered when making comparisons to demonstrate affinity
partitioning and also when selecting partitioning conditions
(ratio of PEG:phosphate phase volumes and required rounds of
phosphate extraction) to render the system more specific for
PEG-protein-DNA complexes.

Figs 2&3 shows the influence of sonication on PEG-
phase yield. As the molecular weight of DNA falls the yield
in the PEG phase increases.

Figure 3 shows the influence of sonication on Peg
phase yield using 1:1 PEG:phosphate phase volume ratio and a
single round of partitioning (circles) or seven rounds with
fresh phosphate phases (squares).

These results are in agreement with those expected on
theoretical grounds since with small molecules the partition
coefficients approach unity (i.e. equal distribution between
the two phases) Muller loc. cit. and the specificity of the
partitioning is lost. Given the known partitioning
coefficients of PEG-proteins Walter loc. cit. which approach
infinity (i.e. 100% in the PEG phase), we anticipated that
the desired affinity-partitioning could still be achieved,
even if DNA was highly fragmented (and this is often
desirable on theoretical grounds - see below), merely by
repeatedly extracting the PEG-phase with additional rounds of
phosphate-phase (figure 2 squares). To limit the number of
rounds required, phase systems can also be constructed with

larg r PO4 than PEG phases.

To establish that additional rounds of phosphate extraction successfully remove small fragments of protein free DNA and leave essentially only protein associated DNA, whole genomic or DNA-protein complex enriched DNA was partitioned with additional rounds of phosphate extraction (a typical example is shown in Figure 3, in which, after 3 rounds of PO_4 extractions very little (0.2%) further DNA was recovered in the PO_4 phase).

Figure 4 shows the effect of additional rounds of extraction with phosphate phase on the recovery of DNA in the PEG and phosphate phases. Results are means$\pm$SEM of 4 independent experiments.

Proteinase K treated aliquots of similarly fractionated samples were processed identically to determine what proportion of the recovered PEG-phase DNA was protein linked (Table I)

TABLE I

START MATERIAL	TREATMENT	SONICATION SECS	ROUNDS	PEG:PO4	YIELD % LOADED
R+ HLWG	PK-	30	4	400:700	0.46%
" "	PK+	"	"	"	0.00%
R+ HLWG	PK-	30	4	400:700	0.70%
" "	PK+	"	"	"	0.00%
R+ HLWG	PK-	30	4	400:700	1.21%
" "	PK+	"	"	"	0.00%
R+ HLWG	PK-	30	4	250:700	0.56%
" "	PK+	"	"	"	0.00%
R+ HLWG	PK-	30	4	250:700	0.36%
" "	PK+	"	"	"	0.01%
R+ TRASK	PK-	0 (DNAse)	5	500:750	7.89%
" "	PK+	"	"	"	0.00%
R+ TRASK	PK-	60	5	500:750	11.42%
" "	PK+	"	"	"	0.00%
R- TRASK	PK-	60	5	500:750	16.38%
" "	PK+	"	"	"	0.60%

In order to establish the extent to which PEG-phase yield is dependent on the attached PEG on the protein or the protein itself, PEG-modified and sham treated DNA-protein complexes were compared, also proteinase K treated samples and protein free DNA derived from aliquots of the same complexes. Protein-DNA covalent complexes (largely topoisomerase-bound DNA Trask loc. cit. prepared by the SDS/KCl precipitation method, were either coupled to MPEG using the activated tresyl-MPEG method described below or sham treated with coupling buffer and untresylated MPEG.

This sham treatment resulted in a significantly lower recovery in the PEG phase (96.3±4.3% reduced yields mean±SEM

of three independent experiments (ranging from 88.8% where 1
round of PO_4 extraction was used, to 100% with 5 rounds of
extraction). Where aliquots of the same PEG-modified DNA-
protein complexes were subjected to to proteinase K treatment
prior to partitioning PEG-phase yields were reduced by
99.7±0.4% and where proteinase K treatment was followed by
phenol chloroform extraction to remove all protein, no DNA
was detected in the PEG-phase in all 3 experiments.

Recoveries of DNA from the two phases varies and is
circa 38.0±8.1% for of load for the PEG phase and 49.7±8.4%
for the PO4 phase using the standard procedure outlined in
methods (using desalting columns and ethanol precipitation).
Results are means±SEM for 6 independent experiments and the
difference is statistically significant ($p < 0.01$; paired t-
test). This will be important in some applications where the
amount of recovered DNA is to be expressed in terms of loaded
DNA and not with respect to a similarly handled control (i.e.
a control dissolved in and then extracted from the relevant
phase(s).

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EXAMPLE 2
USING THE METHOD TO MONITOR LEVELS OF DNA-PROTEIN COMPLEXES
(THIS EXAMPLE IS ALSO RELEVANT TO THE USED OF THE METHOD FOR
THE DETECTION/ASSAY OF DAN BINDING ENZYMES - SEE BELOW)

In order to prepare cells with increasing levels of
two different types of DNA-protein complexes with which to
evaluate the method we exposed cells to: a) VP-16-213
(etoposide) and b) UV radiation which produces covalent
attachment of proteins normally non-covalently bound to DNA.
Figures 5 and 6 show the dose related increment in PEG phase
DNA.

VP-16 (15 min exposure) of whole cells was used to increase
the level of topoisomerase II complexed to DNA. The results
are means±SEM of PEG-phase yields for 8 independent
experiments, expressed with respect to untreated controls.
(Fig. 5).

The amount of DNA complexed to topoisomerase II in
the preparation to be fractionated was varied by exposing
HL60 cells to increasing concentrations of VP16-213. This
drug stablises the enzyme at the stage where it is covalently
bound to DNA (11) and prevents completion of the reaction
(the ligation and dissociation steps). Figure 1a shows a
dose-related increase in DNA recovery from the PEG phase
maximal at 10^{-5}M VP16-213. Phase partitioning was performed
as described below (multiple phosphate extractions were not
used in these experiments). Results are means ±SEM of 8
independent experiments and are expressed as a % of the
amount of DNA recovered without exposure of cells to VP16-
213. In 2/8 experiments the sample was sonicated prior to
fractionation. An increment at 10^{-9}M VP16-213 was detected
in both the sonicated and 3 of the 6 unsonicated samples,
otherwise responses were similar.

This unusual sensitivity of the method is discuss d
further below, as is the heterogeneity of response (revealed

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here as a shoulder on the dose response curve and confirmed
in additional experiments).
UV cross linking was used to increase the level of protein
covalently bound to DNA. The results are means±SEM of 3
independent experiments expressed with respect to untreated
controls. (Fig. 6).

  UV radiation is well known to induce covalent
crosslinking between proteins and DNA. This example is
relevant to the adaptation of the method for the examination
of proteins that bind to DNA non-covalently with the method
(also further discussed in and example below).

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EXAMPLE 3

COMPARISON OF THE SDS/KCL PRECIPITATION METHOD AND THE NEW
TECHNIQUE: IMPROVED RECOVERY OF DNA AND IMPROVED DIFFERENTIAL
RECOVERY BETWEEN DIFFERENTIATING AND UNDIFFERENTIATED CELLS
WHEN USING THE TWO METHODS IN SERIES

We evaluated the performance of the two techniques on
differentiating and undifferentiated cells (Table II). Since
yield with the new procedure is highly dependent on the
average length of the DNA fragments, no definitive yield for
the method can be calculated. With 5 seconds sonication the
new procedure produces a higher yield than for the Trask
procedure. That this occurs despite a much smaller fragment
size (circa 20-40 fold) the 5 second sonicated sample
indicated that the new method recovers many more DNA
fragments. This size differential is illustrated by
comparing the Trask material's DNA molecular weight in Figure
9 (Example 6) with 5 second sonication DNA sample in Figure 5
(Example 2). Since each fragment represents one or more DNA
topoisomerase II cleavage sites the method is much more
efficient with respect to those sites.

TABLE II: COMPARISON OF THE NEW METHOD WITH THE SDS/KCL PRECIPITATION METHOD.

CELL PRE-TREATMENT (HL60 CELLS)	THIS PROCEDURE* (n)	TRASK METHOD (n)
	Yield, % loaded DNA	
	5 SECS SONICATION	
RETINOIC ACID	6.0 ± 3.0 (3)	2.61 ± 0.52 (25)
NONE	6.4 ± 1.6 (3)	2.37 ± 0.48 (25)

* Using 5 seconds sonication and 4 rounds of phosphate extraction (to compensate for the smaller DNA fragment size induced by sonication - see methods).

Although there is independent experimental evidence for DNA/topoisomerase II associations during induction of differentiation, neither technique, used singly, showed significant differences in the amount of DNA recovered from induced and uninduced cells (Table II). This is not surprising, since estimates of the number of DNA breaks (or break clusters) per cell occurring in induced differentiation are low, possibly only 50 per cell and since the modal fragment length with 5 seconds sonication is low, this represents a small proportion of the genome.

We mentioned the theoretical reservations about the SDS/KC1 precipitation method for the preparation of DNA/topoisomerase II complexes, that were pointed out Trask et al elsewhere. His method is only efficient for sites where many topoisomerase molecules are attached to a singl

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strand of DNA. This may in part explain the differences observed here.

It should be noted that given the possible differences in the distribution of topoisomerase cleavage sites at the replication fork and differentiation-associated break sites, the new method may, since it can partition DNA to which a single topoisomerase molecule is attached, be of advantage for the preparation of differentiation site DNA. This does not of course mean that the Trask procedure cannot be used in series with the new technique to produce a two-step fractionation procedure for differentiation-associated topoisomerase cleavage sites, since even if the efficiency of precipitation is low, differentiation sites with one or few topoisomerase molecules are not likely to be less represented in the precipitate than in the supernatant.

As outlined above, the increased yield with the new method, which could reflect more efficient recovery of DNA associated with single topoisomerase molecules, has implications of the use of the method in applications where very few molecules of protein are attached to DNA (e.g. isolated DNA regulatory sequnces are known to contain single topoisomerase II cleavage sites or small clusters of 2-5 sites). Two examples given below corroborate this notion that the new method improves detection of such sites.
1) Experiments where affinity partitioning of very small molecules of DNA was achieved (circa 120-400 bp). Since

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previously published DNAse protection assays show that
fragments of 140 bp are protected at each individual
topoisomerase II cleavage site if digestion is carried out in
the presence of the enzyme, fragments in this size range
should include some that were attached to a single enzyme
molecule (see below).

2) The ability of the method to enrich for differentiation-
specific protein DNA compexes (see below).

We also explored using the two methods in series
(Table III). Here in contrast, in 9 experiments (using 60
sec sonication or 60 sec sonication with additional DNAse
treatment), there was a significant increase in the
proportion of PEG-phase DNA recovered from retinoic acid
treated cells when the start material was SDS/DC1
precipitated ("Trask") DNA. This enhanced recovery of DNA
from the Trask Material from differentiating cells was
dependent on the fragment size selected for the phase-
partitioning, since it was observed with small fragments
(e.g. at 60 seconds sonication, with or without additional
DNAse digestion) but was not observed when using 20 seconds
sonication. With no sonication, virtually all Trask material
(which when prepared avoiding shearing consists mainly of
fragments over 20Kbp) carries to the PEG-phase, precluding
differential recovery.

TABLE III

CELL PRE-TREATMENT

(HL60 CELLS)

	Yield, % whole genomic DNA		Yield % Trask DNA	
	60 SECS* SONICATIONS	20 SECS SONICATION	60 SECS* SONICATION	20 SECS SONICATION
RETINOIC ACID	0.027±0.015 (8)	0.40+0.14 (10)	1.46±0.33 (9)	11.79±2.34 (10)
NONE	0.020±0.008 (8)	0.57+0.28 (10)	0.52±0.17 (9)	16.09±6.20 (10)
p (paired t-test)			0.029	
	N.S.	N.S.	SIGNIFICANT	N.S.

*With additional DNAse digestion in 3 experiments

The differential recovery suggests several possibilities: an increase in the ratio of protein-bound DNA to free in SDS/KCL precipitates from differentiating versus undifferentiated cells (anticipated on the basis of the known actions of DNA topoisomerase II in differentiation) however we cannot exclude: a qualitative change in the types of proteins bound vis a vis their accessible lysyl residues for PEG-modification; or a decreasse in the average DNA fragment size of the SDS/KCL precipitates (since with longer fragments it is more difficult to overcome the affinity to the phosphate phase). This latter hypothesis seems unlikely, since, although high resolution electrophoresis of SDS/KCL precipitates has not yet been performed, no dramatic reduction of these fragments below 20Kbp seems to be occurring in differentiated cells.

For increased recovery to occur from the SDS/KCL precipitates without an significant increase in recovery when results are expressed with respect to the start (whole

g nomic) material, does not necessarily indicate that other
(non-differentiation associated) protein/DNA covalent
interactions may have been lost during induction. The second
round recoveries are only a very small fraction of the
intermediate recovery and errors in relating the SDS/KCL
recovery to the whole genomic DNA could readily have masked
any increment.

EXAMPLE 4

USING SONICATION TO REDUCE DNA LENGTH AND IMPROVE ENRICHMENT
OF THE DNA MOST CLOSELY ASSOCIATED TO THE PROTEIN AND
RECOVERY OF COMPLEXES CONTAINING SINGLE PROTEIN-BINDING
SITES.

Sonication and other methods to fragment DNA have two
influences on the method. Opposing forces act on the DNA-
protein complexes. If the DNA attached to a single molecule
is long enough it could, via its affinity for the phosphate
phase, retain the complex in that phase. Shortening the DNA
will reduce the likelihood of this occurring. The second
motive for shortening the DNA is if one wishes to recover the
DNA at the protein binding site with little contamination by
adjacent DNA.

1) The effect of sonication on the partition coefficient of
protein free DNA

For the reasons outlined above sonication is
sometimes employed to shorten the average length of DNA
attached to proteins. It is known however that reducing the
length of DNA alters the partition coefficient, reducing the
tendency of DNA to remain in the phosphate phase and leading
to a more equal distribution between the two phases. The
improved enrichment of protein-bound DNA in the PEG phase
(enrichment with respect to the DNA-protein binding sites)
achieved by sonication will thus be to some extent
counteracted by this change since additional protein-free DNA
will partition to the PEG phase with increasing sonication
(Figs. 2 and 3).

Unlike the PEG/protein/DNA complexes which will have
a very high affinity for the PEG phase, the protein free DNA
of the PEG phase is extractable by additional rounds of
partitioning with fresh phosphate phases or by counter
current distribution (i.e. chromatographic separation in a
multiple phase partitioning apparatus). The experiment

described in Example 1 and Fig. 4 demonstrates the efficiency
of additional rounds of extraction with phosphate phase in
removing PEG-phase DNA after sonication.

2) Shortening DNA length of DNA protein complexes reduces the DNA recovery from the PEG-phase

Sonication reduces the average length of the DNA in the DNA/protein preparation and concomitantly reduces the DNA partitioning to the PEG phase. Using the protocol in methods and 5 seconds sonication PEG-phase DNA was reduced to $32\pm17\%$ of values in parallel experiments of unsonicated protein-linked DNA (using 4 different types of samples i.e. drug treated and controls).

This modification of the method may be important in achieving detection of complexes where there is only a single isolated protein molecule bound to a section of DNA. It is also potentially useful in applying the method to cloning of DNA at protein binding sites, because sonication removes adjacent DNA.

EXAMPLE 5

ESTABLISHING THAT DNA PROTEIN COMPLEXES WITH A SINGLE ATTACHED TOPOISOMERASE II CAN BE PARTITIONED USING THE METHOD

Figure 7 shows an example of affinity partitioning of small PEG/protein/DNA complexes. DNA/protein complexes were sonicated for 60 seconds to fragment the DNA. Topoisomerase II is know to protect DNA fragments of circa 140 base pairs in DNAse protection assays. Thus fragments of this size are unlikely to have more than single molecule of enzyme attached to the DNA.

This experiment shows small fragments (mainly between molecular weight markers of 564 and 125 bp) recovered from the PEG-phase after partitioning. That these fragments are not due simply to the reduced tendency of small DNA molecules to remain in the phosphate phase (the problem mentioned above) is demonstrated by a control sample which was treated with proteinase K before the coupling step (coupling PEG to the protein/DNA complexes).

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Because of the anticipated very small DNA fragment sizes not only were additional rounds of phosphate extraction used here but the ratio of volumes of the two phases was altered to PEG:PO_4 250:750ul (3 changes of 750ml PO_4 were used).

This experiment is relevant to the claim that the new
technique should improve the detection of differentiation-
specific topoisomerase II DNA complexes, some of which we
know to be relatively isolated in the genome (see Example 7
below).

EXAMPLE 6

USING NUCLEASES TO REDUCE DNA LENGTH AND IMPROVE ENRICHMENT
OF THE DNA MOST CLOSELY ASSOCIATED TO THE PROTEIN AND
RECOVERY OF COMPLEXES CONTAINING SINGLE PROTEIN-BINDING
SITES.

We have also established that nucleases (e.g. DNAse
and restriction endonuclease) can be used to reduce DNA size
of the DNA/protein complexes. DNAse digestion of DNA protein
complexes prepared by the Trask method, using the protocol
described in methods, after 10-20 minutes produces fragments
comparable to 60 seconds sonication (Figure 8). In a similar
preparation the protein "protects" a low molecular weight
band of DNA from digestion (inset in Fig 8): lanes 1 & 2 = no
DNAse; lanes 4-6 = 1,5,10 min DNAse digestion after
proteinase K (the DNA is unprotected and largely digested);
lane 7 = HindIII/EcoR1   DNA; lanes 8-10 = 1,5,10 min DNAse
digestion of protein/DNA complexes (a low molecular weight
band of DNA circa 130 bp is protected). This method can also
therefore be used to reduce DNA size for partitioning either
before the treslation step (Figure 9), or after the first
round of partitioning (Figure 10) before subsequent
partitioning or DNA cloning. The protein K digested control
in Fig 9 demonstrates that the recovered low molecular weight
DNA is the result of affinity partitioning since none is
recovered if the PEG-protein is first removed from the DNA by
proteinase K digestion.

Since the attached protein "protects" the attached
DNA from digestion by DNAse, the method can be used to
prepare samples enriched for such fragments. This has

applications in the analysis of DNA protein binding sites
using the DNAse protection principle.

     As might be expected on the basis of the small
fragment size produced by 60 second sonication of DNA/protein
complexes, an additional partitioning step after DNAseI only
produced a modest reduction in yield of DNA from the PEG
phase. Result show yields from Trask preparations from
retinoic acid induced and uninduced HL60 cells and are
means±SEM for three experiments using both DNase and 60
seconds soication, versus the mean±SEM fpr 5 experiments
using sonication alone.

PEG-Phase yields from Trask preparations comparing three
experiments using both DNase and 60 seconds sonication,
versus 4 experiments using sonication alone are shown in Fig.
10. A modest reduction in yield is seen with material from
both retinoic acid and uninduced HL60 cells.

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EXAMPLE 7

USING THE METHODS TO ENRICH FOR DIFFERENTIATION-SPECIFIC
DNA/PROTEIN COMPLEXES.

a) Demonstration of recovery of quantitatively different
amounts of DNA from SDS/KCL precipitates from differentiating
cells

        We know from previous studies that retinoic acid
induces DNA topoisomerase II to perform DNA breakage-reunion
reactions shortly after the induction of differentiation.
This can be demonstrated in several ways (e.g. nucleoid
sedimentation, the alkaline filter elution technique and the
fluorescent alkaline DNA unwinding - FADU- technique) and is
inhibited by DNA topoisomerase II inhibitors (13). None of
these methods is however suitable for the purification of
these complexes. The Trask procedure as mentioned above, is
likely on theoretical grounds to be inefficient for isolated
protein molecules attached to DNA. It also does not in
practice recover greater amounts of DNA/protein complexes
from retinoic acid treated cells than from controls (see
Examples 3 above). The Trask technique is unlikely therefore
to be providing a significant enrichment of this type of
DNA/protein complex vis a vis other complexes (the technique
is known to be highly efficient for DNA/topoisomerase II
complexes from DNA replication forks).

        In order to examine whether the new technique can
enrich for and/or recover sufficient quantities of the
retinoic acid induced complexes for their presence to be
detected, we employed the two "short fragment" modifications
described above: either 60 seconds sonication, or sonication
plus an additional round of partitioning after DNAse
treatment. The start material in each case was a DNA/protein
complex preparation precipiated by the Trask method.

        As Figure 8 shows, in 7/9 experiments a higher
proportion of the start material was recovered from the

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retinoic acid treated material. This difference is
statistically significant (p = 0.029; paired t-test).

Differential yields from Trask material when prepared from
differentiating and undifferentiated HL60 cells are shown in
Figure 11.

b) Demonstration that specific DNA sequences with
differentiation-associated attachment to prtein are enriched
by the procedure

To test whether differentiation involves specific DNA
sequences, we obtained highly purified Topo II-associated DNA
from RA induced differentiating cells, labelled and
hybridised this DNA to DNA enriched for, or depleted of, Topo
II-bound DNA from both undifferentiated HL60 cells and cells
induced to differentiate by RA or phorbol ester.
Differential hybridisation demonstrated that the isolated DNA
is enriched for specific sequences. Differential
hybridisation was also seen with myc and fos probes
suggesting that Topo II cleavage occurs near these genes.
This is, as far as we are aware, the first demonstration that
Topo II interacts at specific (or limited) sites in the
genome during the induction of differentiation.
It is important to note that although enhanced recovery is
seen when topoisomerase II is arrested at the covalent
complex stage by VP-16 (etoposide), this agent is not used in
the following experiments since it is known to influence
cleavage site specificity.
Since opposing forces act in the phase partitioning system
(longer DNA fragments oppose affinity partitioning)
theoretically the maximum efficiency of recovery of cleavage
site DNA will occur with the lowest possible fragment length.
To this end we have used DNAse digestion to remove protruding
DNA (topoisomerase are known to protect circa 140bp of DNA as

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they act). This experimental detail is particularly
important for the recovery of differentiation specific breaks
sites since it will reduce the presence of contaminating
repetitive sequences.

DNAse digestion of DNA/Topo II complexes prepared by the
Trask method for 1, 2 and 5 minutes demonstrates a clear
protected band of DNA and this material can be affinity
partitioned (see Examples above).

Since we have demonstrated that affinity partitioning (as
opposed to the Trask method) can recover DNA attached to a
single topoisomerase molecule, the new method has theoretical
advantages for the preparation of highly enriched DNA from
differentiation cleavage sites (whereas the Trask technique
is most efficient for replication fork DNA).

To test whether the Topo II cleavage sites are at specific
sites in the genome we prepared sets of 4 different DNA
samples:-

1) Topo-associated DNA from differentiating cells

2) Topo-associated DNA from undifferentiated cells

3) DNA depleted of Topo cleavage sites from differentiating
   cells

4) DNA depleted of Topo celaves sites from undifferentiating
   cells

Using dot blot hybridisation to DNAse protected Topo-
associated DNA from differentiated cells the anticipated
patterns of hybridisation (if specific DNA sequence become
associated with topoisomerase or other proteins during
differentiation and are thus recovered by the method) are as
follows:-

1) +++ (enriched relative to undifferentiated control)

2) ++

3) + (depleted relative to undifferentiated control)

4) ++

Differential hybridisation shows that specific sequences

b come protein-bound during retinoic acid induced
granulocytic differentiation (Figure 12).

Differential hybridisation is also observed when the filter
DNA is prepared from phorbol ester induced cells (Figure 13).
Thus some sites at least are shared between monocytic and
granulocytic differentiation. This results indicates that
the DNA sequences recovered by this method may have broader
applicability than just defining differentiation events in
one specific cell lineage and inducer system.

c) Demonstration that the differential hybridisation is reproducible

Fig. 14 shows that similar differential hybridisation
is achieved with independently prepared filter and probe
samples (2 probe and 6 filter samples). PMA hybridisation
also showed reproducible differential hybridisation.
In 10/10 hybridisations there is a more intense signal from
the R+ than R- DNA as shown in Figue 14.

d) Demonstration that genes with known changes in expression during haemopoietic cell differentiation are fractionated by the method (and demonstation that this is due to sequences in or around the gene undergoing topoisomerase association)

This differential hybridisation is also observed when the
filter are probed with both myc and fos probes.

The succes in enriching for two genes whose expression is
known to be modulated during haemopoietic cell
differentiation (c-myc and c-fos) suggests that the new
technique provides a strategy to search for hitherto unknown
differentiation genes. Break-site DNA is currently being
cloned for identification of "differentiation genes" and
putative regulatory sites.

These findings indicate that differentiation associated Topo
II cleavage r actions (and/or other DNA protein linking

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events) take place in or near the myc and fos genes during induced granulocytic differentiation. Since differential hybridisation was lost for myc when novobiosin was added (see Figure 16 other DNA protein linking events seem unlikely. Scanning densitometer traces of Fig. 16 show differential hybridisation to the myc probe by R+ and R- DNA is lost when R+ cells are pretreated with novobiocin (an inhibitor of Topo II); lines, A, B and C respectively.

Since the differential hybridisation observed between DNA from differentiating and undifferentiated cells was abolished by novobiocin a known inhibitor of Topo II. This suggests that covalent attachment to this enzyme was, although not necessarily responsible for all DNA recovered, responsible for the recovery of DNA influencing the differential hybridisation.

Quantitative analysis of the amount of DNA recovered from differentiating and undifferentiated cells suggests that the DNA responsible for the differential hybridisation may be particularly sensitive to the inhibitory action of novobiocin (Figs. 17 and 18).

Novobiocin induced a modest reduction in the recovery of "Trask" DNA, but only in differentiating cells, as shown in Fig. 17.

Novobiocin produced no significant decrease in the amount of DNA recovered by the combined Trask/new procedure, neither in cells induced with retinoic acid nor in undifferentiated cells as shown in Figure 18.

e) Demonstrations that DNA flanking stably expressed (differentiation unmodulated) genes is not enriched by the method.

Similar hybridisation experiments to those for myc and fos were performed using a tubulin gene probe (Cambridge BioSciences). Figures 19 and 20 show that with neither retinoic acid nor phorbol ester induced cells is there

differentiatial hybridisation to the tubulin probe with PEG-
phase recovered protein associated DNA.

Lack of differntial hybridisation with R+ and R- DNA
(under similar conditions to those used for myc and fos) when
the gene probe is tubulin is shown in Fig. 19.

Lack of differential hybridisation with PMA+ and PMA-
DNA (under similar conditions to those used for myc and fos)
when the gene probe is tubulin is shown in Fig. 20.

EXAMPLE 8

<u>USING THE MEHTOD TO PREPARE DNA FOR CLONING AND
DEMONSTRATIONS THAT SOME CLONES SHOW DIFFERENTIAL
HYBRIDISATION TO DIFFERENTIATION-ASSOCIATED TOPOISOMERASE
CLEAVAGE SITE DNA</u>

Since one use of the method is to prepare specific
DNA sequences for cloning we have cloned DNA from retinoic
acid induced DNA/protein complexes (prepared as described
above with either sonication or DNAse digestion) to
demonstrate that the method produces DNA suitable for cloning
procedures.

Two different cloning techniques were evaluated, but
in principal any standard technique could be used.

A)   The homopolymer tailing technique was used to
introduce the prepared DNA fragments into dCTP tailed Plasmid
(pUC8). This method was selected because no differential
handling of topoisomerase cleavage sites, sheared ends or
restriction sites is involved (we wanted to avoid differences
in efficiency of processing fragments with and without the
topoisomerase staggered cleavage sites). Transformation
followed by growth of plasmid in, and recovery from DH5 E.
coli, demonstrated that recombinant plasmids were produced.
However the eficiency of this method was relatively low.

Scanning densitometry of agarose gel electrophoresis
of plasmids from which DNA inserts have been cleaved by
EcoRl/HindIII digestion as shown in Fig. 21.

B)   The second strategy was to infill the overhang
created by the topoisomerase cleavage reaction using the
Klenow polymerase and to attach Sal I linkers. After
digestion with EcoRI and SAl I, the DNA was ligated into
pUc18, similarly digested, and transformed into E. coli DH5
cells.

Clones were screened for differential hybridisation
to R+ R- and whole genomic probes (i.e. using a similar

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strategy to that used above), to determine DNA inserts which
represent DNA sequences preferentially associating with
topoisomerase during the induction of differentiation. A
sample of 23 of the first 298 clones obtained shown in
Figures 22 and 23.

The combined hybridisation signal (absorbance per
mm2) for three hybridisations to measured amounts of DNA from
clones is sh shown in Fig. 22 R+ and R- probes were as for
the differential hybridisation experiments above, WG was
labelled whole genomic (unfractionated) HL60 cell DNA.

The hybridisation signals (absorbance per mm2) for
three hybridisations, given in fig. 22 are expressed as a
proportion of the combined hybridisation signal in Fig. 23.

These results demonstrate not only that the material
can be cloned, but that a proportion of resulting clones have
specific DNA sequences that become protein associated as a
result of inducing differentiation. Since topoisomerase
modulates gene expression by a mechanism requiring it to act
in the vicinity of the affected gene, these DNA fragments
will be in or near differentiation genes modulated by
topoisomerase II.

EXAMPLE 9

USING THE METHOD FOR THE DETECTION/ASSAY OF TOPOISOMERASE
INHIBITORS

As implied by the dose response curves above in Example 2,
the method can be used to quantitate the complexing of
topoisomerase to DNA induced or inhibited by different
classes of topoisomerase II inhibitors.

The method is very sensitive for the detection of VP-
16, detecting a 326 ± 100.25 %increase in PEG phase DNA at
10^{-9}M VP16 (means $\pm$SEM of 10 experiments using either
undifferentiated or differentiation induced HL60.   An
increment (maximum 950%) was detected in 7/10 experiments.
Increments in PEG phase DNA in the majority of samples at
10^{-9} VP16-213 as shown in Fig. 24.
Uninduced cells were exposed to VP16 for 15 min.   Induced
cells were exposed to retinoic acid (10^{-6}M) for 70 minutes
and simultaneously to the VP16.   As mentioned in Example 2
above sonication influenced the proportion of samples showing
an increment and thus this and other facets of the method may
have to be optimised to yield the most sensitive assay for
any individual system.

This result indicates that the new method may be much
more sensitive to the action of inhibitors than other assays
which usually detect VP16 only at the range 10-7 to 10-5M.

This sensitivity is not resricted to the
epipodophyllotoxin type topoisomerase II inhibitors and
similar results can be obtained with interclators.

The effect of m-AMSA, an intercalator, on PEG phase
yield is shown in Fig. 25.   Since the inactive analogue
o-AMSA (which does not significantly inhibit topoisomerase
II) influenced phase partitioning results, yields were
corrected with respect to an o-AMSA control.

EXAMPLE 10

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USE OF THE METHOD FOR THE DETECTION OF HETEROGENEITY IN
RESPONSE TO INHIBITORS

After induction of cells by retinoic acid for 70 minutes and
concomitant exposure to VP16 (the latter at the doses shown),
the enhancement of PEG phase yield is clearly biphasic. The
results in Figures 24 and 25 shown two independent
experiments. Although only preliminary data, a similar
"shouldering" effect is seen in unstimulated cells. This
observation may be related to the observation in cell systems
that VP-16 inhibits differentiation at $10^{9-}M$ whereas it
inhibits proliferation only at $10^{7-}M$ an above, indicating
functional heterogeneity of the enzyme involved in the two
processes.

The method thus provides a method for discriminating
topoisomerases with inherent or imposed different
susceptibilities to inhibitor.

The effect of VP-16 on PEG phase yield after retinoic
acid treatment is shown in Fig. 26.

EXAMPLE 11

USING THE METHOD TO EXAMINE NON-COVALENT
ASSOCIATIONS/DISSOCIATIONS BETWEEN PROTEINS AND DNA

If a crosslinking step is used, in theory, changes in the
association/dissociation of proteins that do not normally
covalently link themselves to DNA may be assessed by the
method. We have previously established that during retinoic
acid induction of haemopoietic cell differentiation there is
an increase in the amount of protein associated with DNA. To
demonstrate whether the new technique could detect this,
cells were exposed to retinoic acid for the periods shown and
were than subject to UV illumination to crosslink protein to
DNA. Although cross linking is a stochastic process, any net
increase in protein association should be revealed in an
increase in the crosslinking of proteins to DNA. Since HL60
are notoriously susceptible to stress and to shock-induced

differentiation, sham treated (handling) controls were used
and results for each time point were expressed with respect
to these controls.

As figure 27 shows, there is a significant increase
in PEG-phase protein associated DNA peaking at 70min exposure
to retinoids. Since a similar increment was not observed
without a UV crosslinking step (cf. Table II above, where
there is no significant difference in PEG phase DNA after 70
min retinoic acid treatment), this increase is concluded to
be due to cross linking of non-covalently bound proteins
which do not contribute significantly to the PEG-phase yield
under the standard conditions.

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CLAIMS

1. A process for separating covalent DNA/protein complexes from non-covalent DNA/protein complexes and unbound DNA comprising the steps of:

(i) treating the DNA/protein complexes with a reactive derivative of polyethyleneglycol (PEG) and

(ii) subjecting the product of step (i) to phase partition between an aqueous PEG solution phase and an aqueous phosphate solution phase.

2. A process according to claim 1 compising the further step of:

(iii) recovering covalently bound DNA/protein complexes from the aqueous PEG phase.

3. A process according to claim 1 or claim 2 wherein the reactive derivative of PEG is 2,2,2-trifluoroethanesulphonyl monomethoxy polyethyleneglycol (TMPEG).

4. A process according to any one of claims 1 to 3 wherein the covalently bound DNA/protein complex is a DNA/topoisomerase complex.

5. A process according to claim 4 wherein the DNA topoisomerase is DNA topoisomerase I and/or II.

6. A proces for purifying DNA comprising or associated with an inducible gene wherein induction of transciptional activity is accompanied by the action of one of more enzymes forming covalent intermediates with DNA comprising separating a covalent DNA/enzyme complex by a process according to any one of claims 1 to 5.

7. A process for purifying DNA comprising a protein binding site comprising, where necessary, effecting covalent binding of the protein to the DNA at the protein binding site, and separating a covalent DNA/protein complex by a process according to any one of claims 1 to 5.

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8. A process for assaying DNA topoisomerase activity comprising separation of DNA/topoisomerase complexes by a process according to claim 4 or claim 5.

9. A process for assaying DNA topoisomerase cleavage-site specificity comprising separating covalent DNA/topoisomerase complexes by a process according to claim 4 or claim 5.

10. A process for producing DNA comprising a protein binding site which process comprises forming a covalent complex of a DNA fragment comprising the protein binding site and a protein which binds to the site, where necessary reducing the length of the DNA fragment by digestion using a DNAase enzyme and separating the resultant DNA/protein complexes by a process according to any one of claims 1 to 5.

11. A process for assaying DNA-protein cross-linking agents comprising separating covalent DNA/protein complexes by a process according to any one of claims 1 to 5.

    12. A process for assaying an inhibitor of a
nicking/closing enzyme comprising separating covalent
DNA/enzyme complexes by a process according to any one of
claims 1 to 5.

Fig. 1.

Panel 1: $CH_3(OCH_2CH_2)_nOH + ClSO_2CH_2CF_3$

MPEG   TRESYL CHLORIDE

↓ ↘ HCl

$CH_3(OCH_2CH_2)_nOSO_2CH_2CF_3$

TRESYLATED MPEG
ACTIVATION OF MPEG
BY TRESYL CHLORIDE

Panel 2: NH_2 NH_2 $HOSO_2CH_2CF_3$

$CH_3(OCH_2CH_2)_nHN-$

MPEG-MODIFIED PROTEIN

Panel 3: (▷ TMPEG)

CONFORMATIONAL CHANGES
DISPLACE NON-COVALENTLEY
BOUND PROTEINS

Panel 4: PEG   PO$_4$

PHASE PARTITIONING

Panel 6: PROTEINASE K

TOPOISOMERASE-BOUND DNA
IS RECOVERED
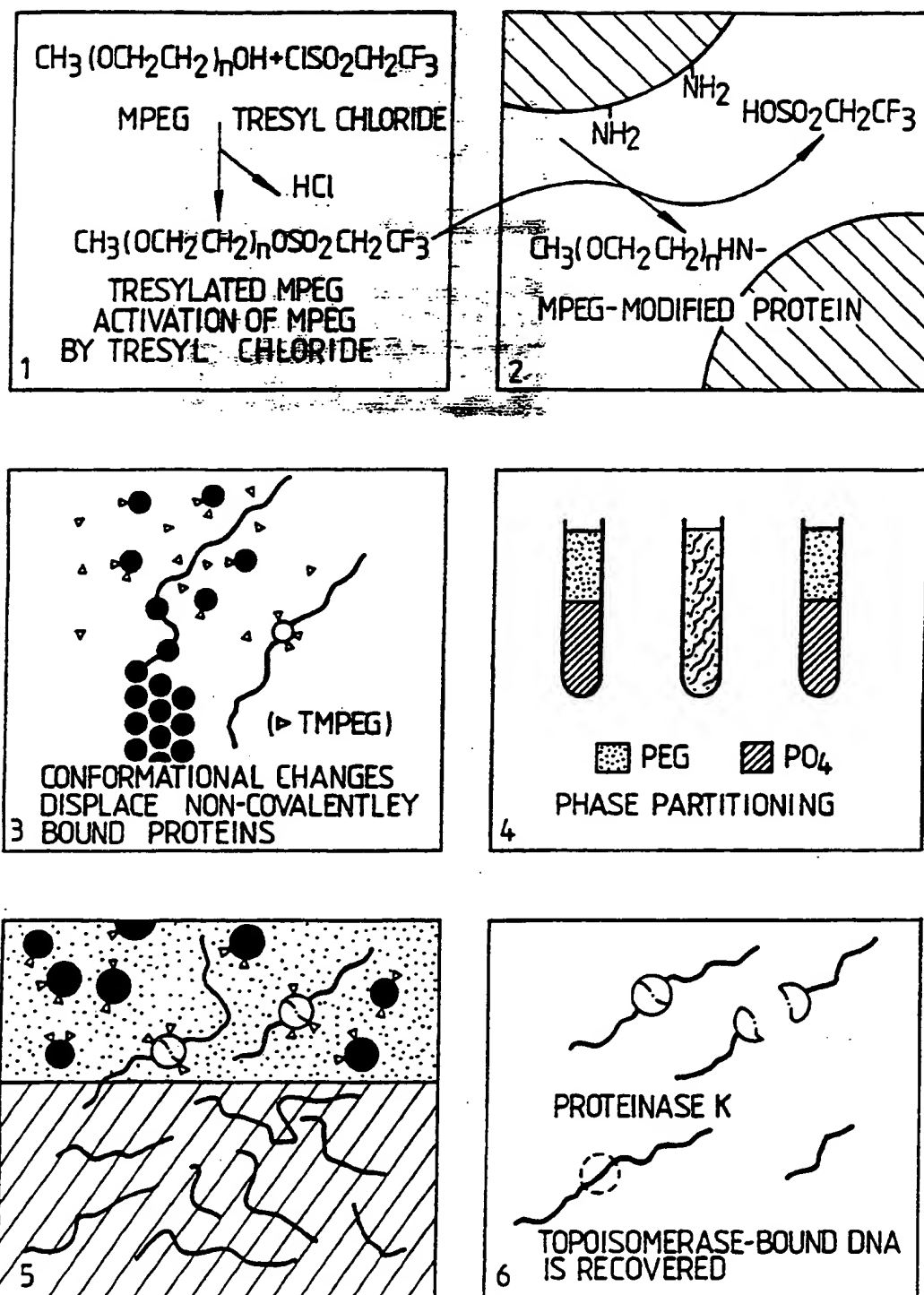
Fig. 2.

THE EFFECT OF SONICATION ON THE MOLECULAR WEIGHT OF SARMON SPERM DNA. (12μ PEAK TO PEAK AMPLITUDE, 20kHz 150W)

MIGRATION ⟶

Hind III / ECoR1 DIGESTED λDNA MOLECULAR WEIGHT MARKERS (in kBp)

0·56   0·98 0·83   2·02 1·58   5·14   21·2

60 SECONDS
45 SECONDS
30 SECONDS
10 SECONDS
5 SECONDS

0 SECONDS
GEL SHADOW
WELL
WELL

ABSORBANCE ⟶
1·0A
0
0

3/18

Fig. 3.

EFFCET OF SONICATION OF PROTEIN-FREE DNA
PARTITIONING TO THE PEG RICH PHASE

■ 7 ROUNDS EXTRACTION
● 1 ROUND EXTRACTION

% DNA IN PEG PHASE (%TOTAL LOADED DNA)

SONICATION TIME (SECONDS) 20 kHz, 12u AND 150 W.

Fig. 4.

THE EFFECT OF EXTRA ROUNDS OF PHOSPHATE
PHASE EXTRACTION ON RECOVERY OF DNA FROM
THE PEG PHASE

% TOTAL DNA RECOVERED

ROUNDS OF PHOSPHATE PHASE EXTRACTION

1st PO4   2nd PO4   3rd PO4   4th PO4   PEG PHASE

Fig. 5.

EFFECT OF VP16 ON PEG-PHASE YIELD
MEANS OF 8 INDEPENDENT EXPERIMENTS

Fig. 6.

EFFECT OF UV ON PEG-PHASE YIELD
MEANS OF 3 EXPERIMENTS

Fig. 7.

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Fig. 8.

THE EFFECT OF DNaseI ON THE MOLECULAR WEIGHT
OF PROTEIN ASSOCIATES (TRASK) DNA
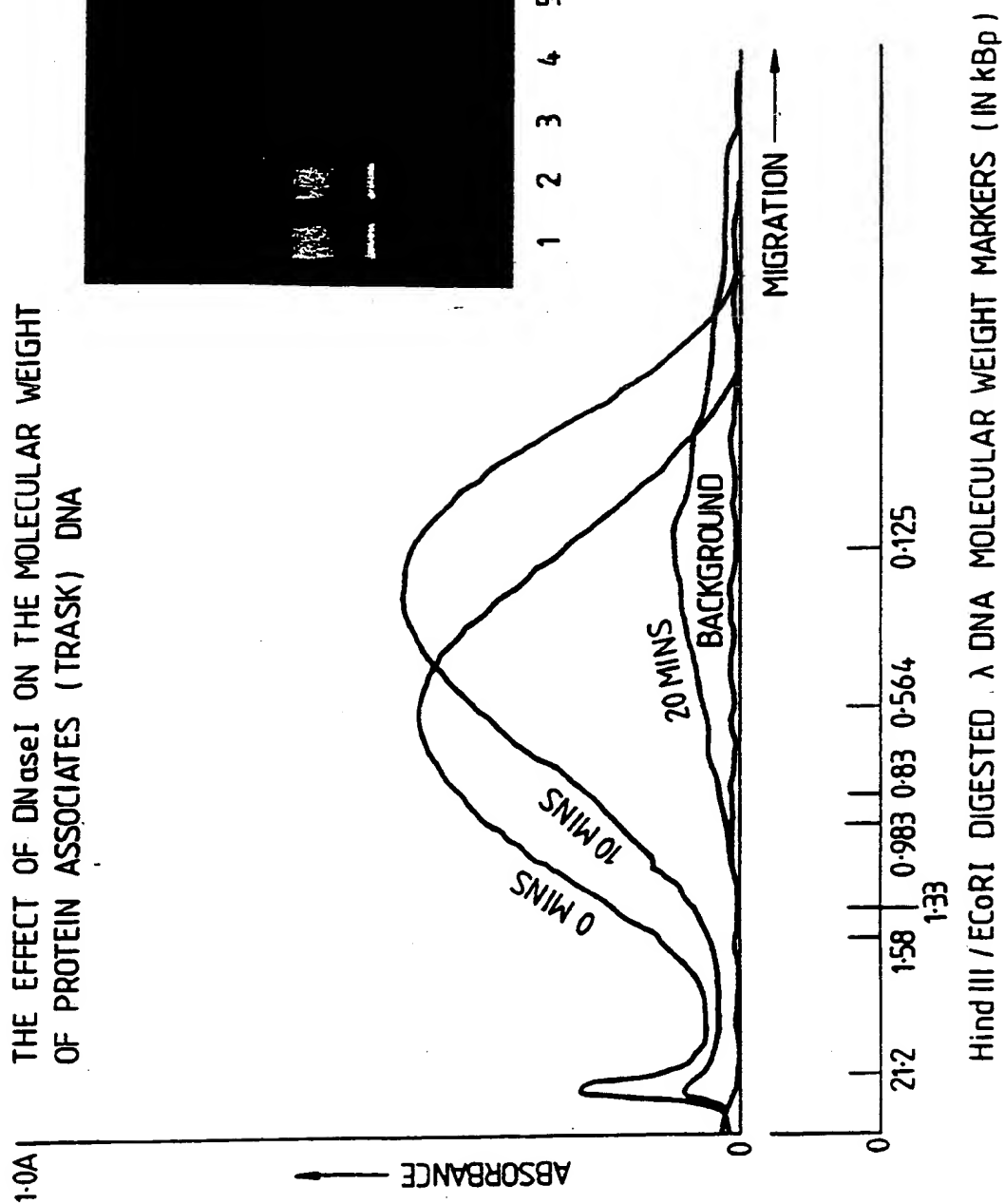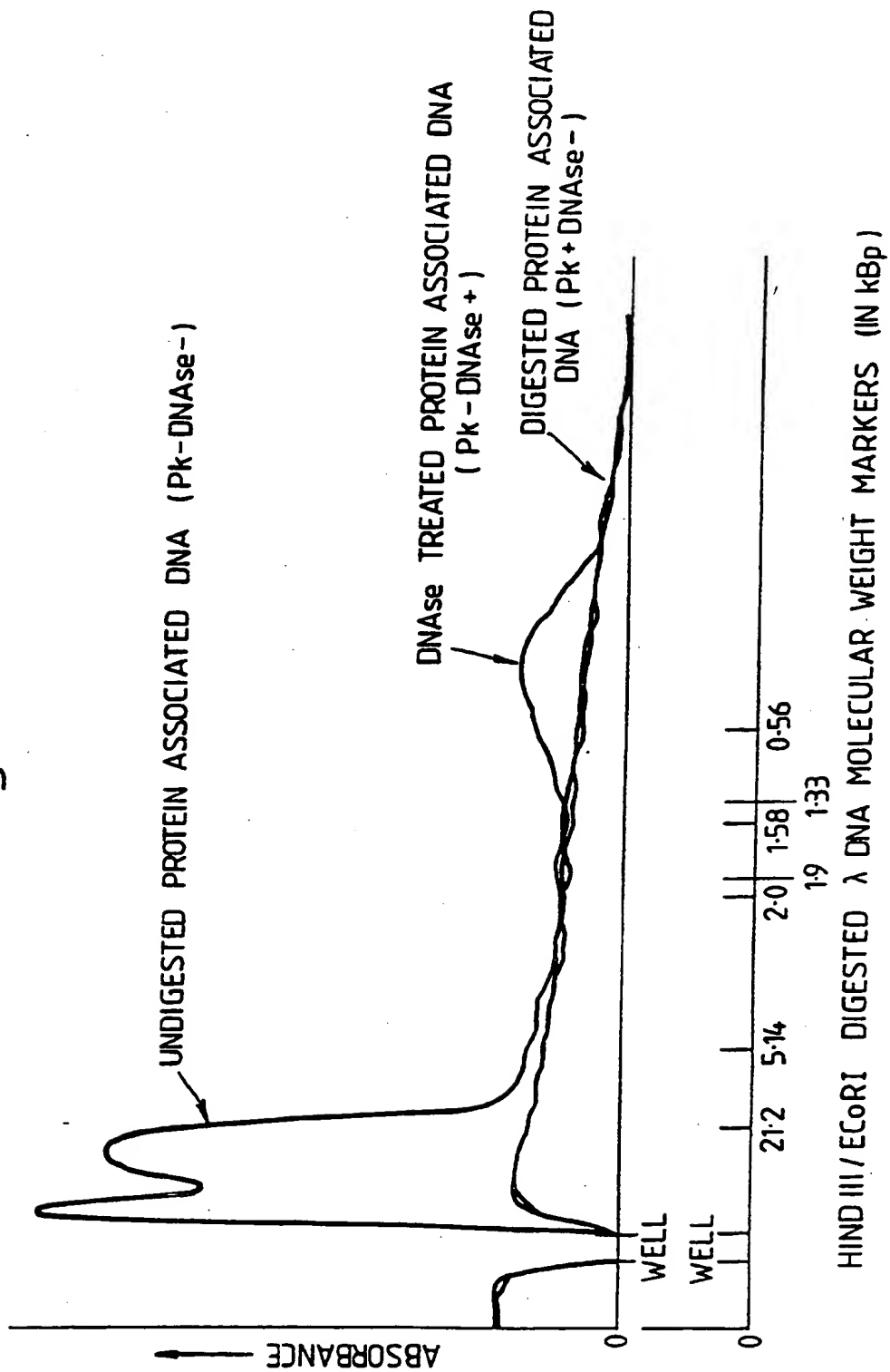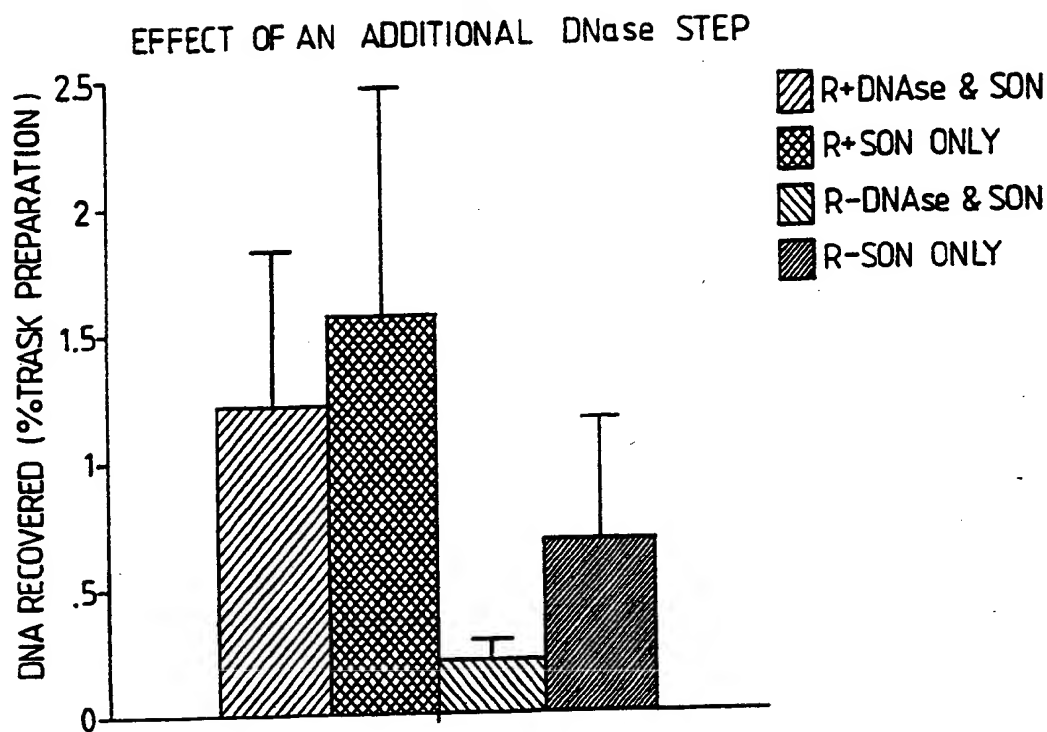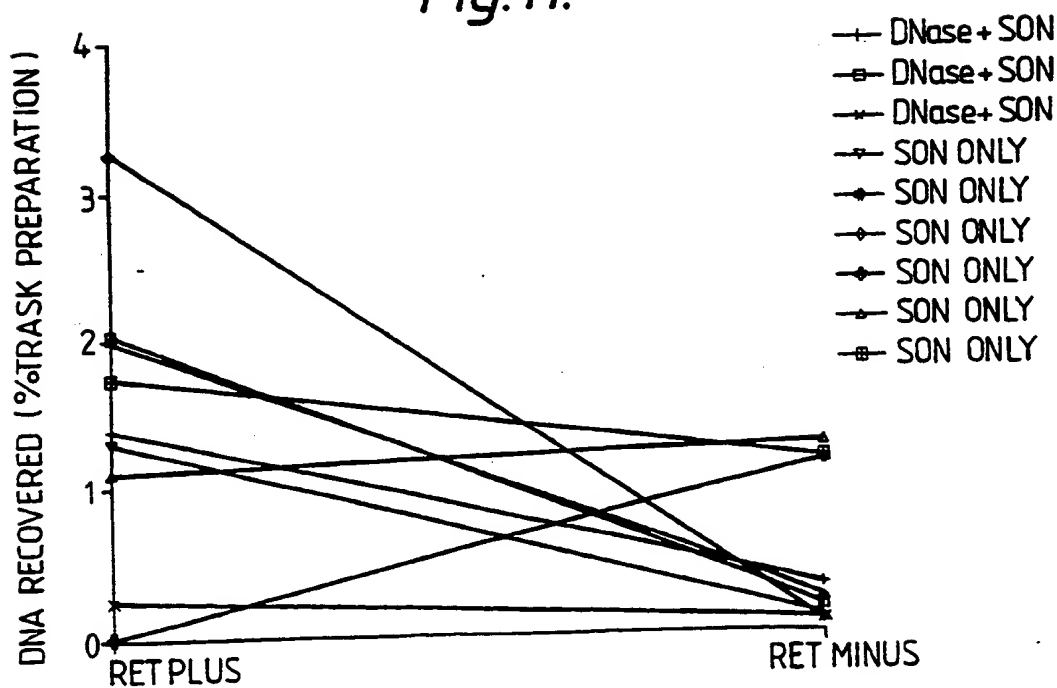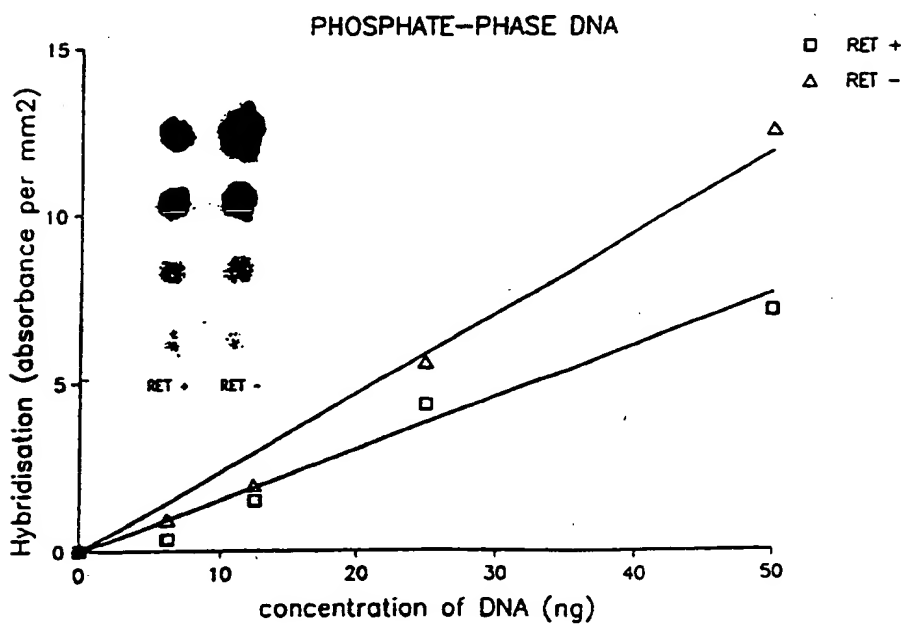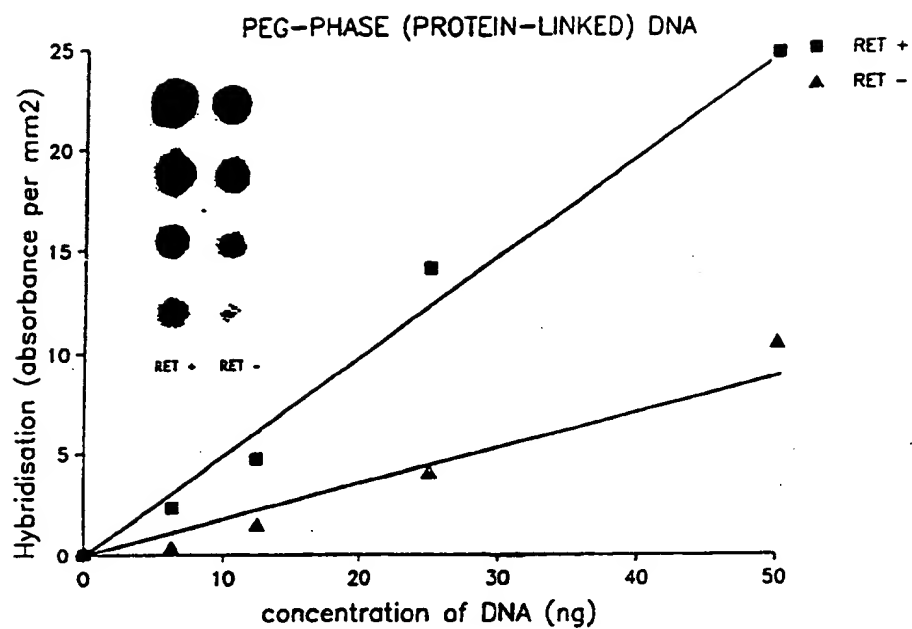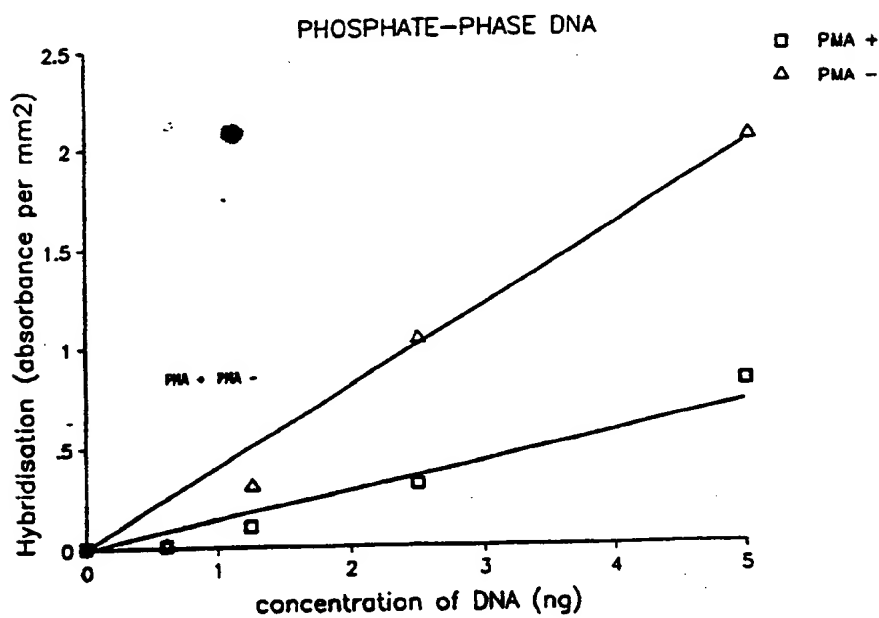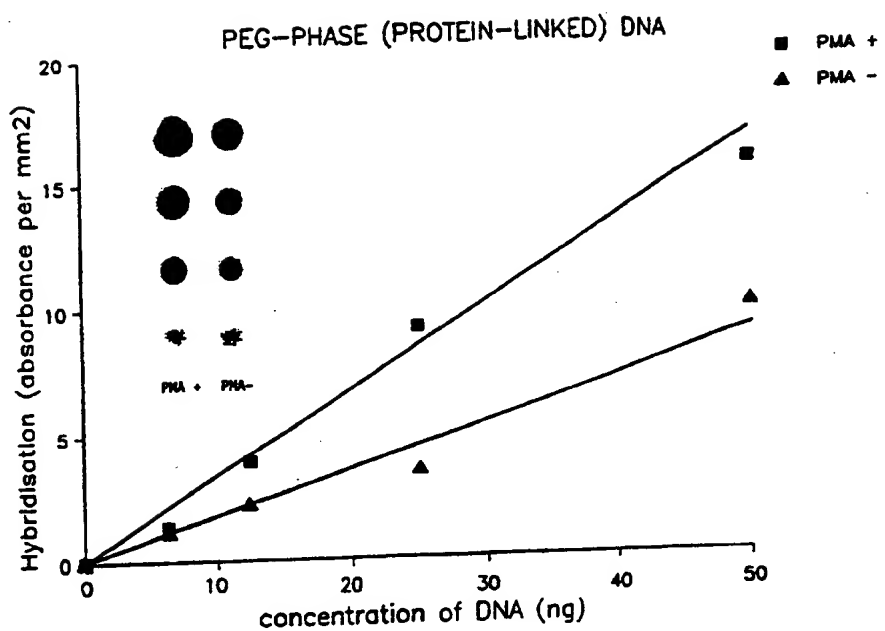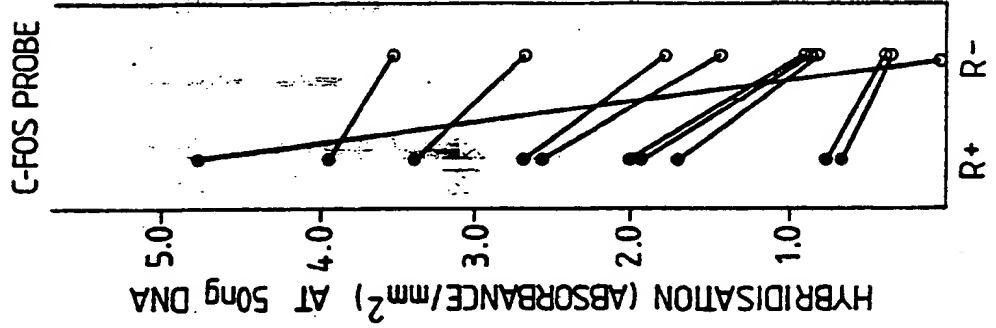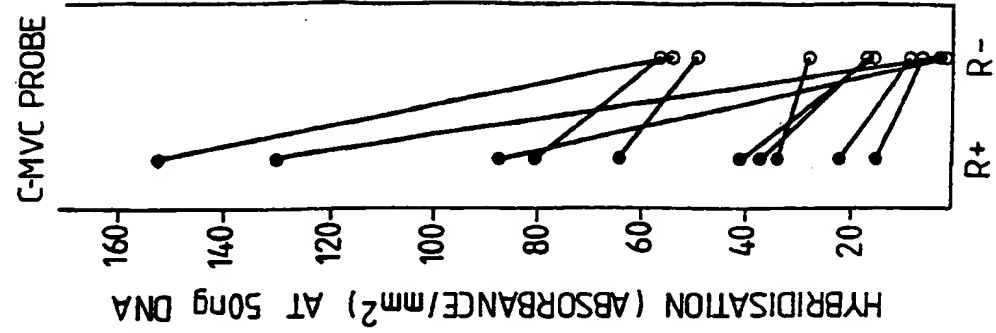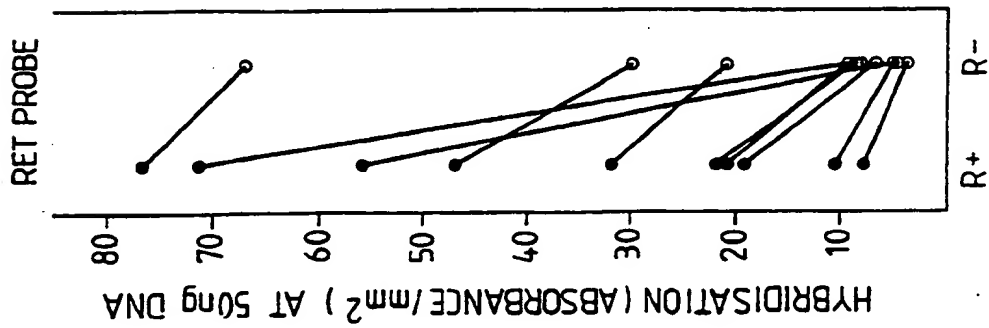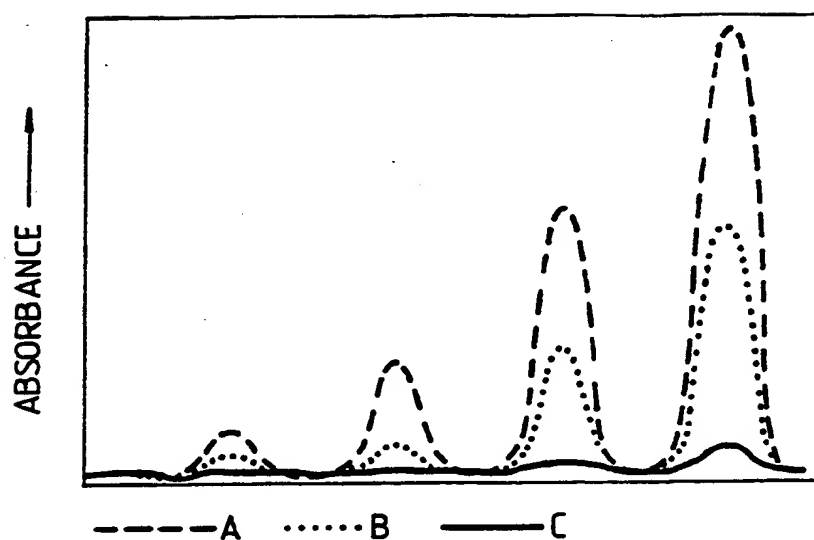
Fig. 9.

Fig. 10.

EFFECT OF AN ADDITIONAL DNase STEP

Legend:
- ▨ R+DNAse & SON
- ▩ R+SON ONLY
- ▨ R-DNAse & SON
- ▨ R-SON ONLY

Fig. 11.

Legend:
- —+— DNase + SON
- —▭— DNase + SON
- —▼— DNase + SON
- —▽— SON ONLY
- —◆— SON ONLY
- —◇— SON ONLY
- —◆— SON ONLY
- —△— SON ONLY
- —▣— SON ONLY

RET PLUS   RET MINUS

Fig.12.

PEG-PHASE (PROTEIN-LINKED) DNA

PHOSPHATE-PHASE DNA

Fig.13.

Fig. 15.

C-MVC PROBE

HYBRIDISATION (ABSORBANCE/mm²) AT 50ng DNA

R+   R-

160
140
120
100
80
60
40
20

C-FOS PROBE

HYBRIDISATION (ABSORBANCE/mm²) AT 50ng DNA

R+   R-

5.0
4.0
3.0
2.0
1.0

Fig. 14.

RET PROBE

HYBRIDISATION (ABSORBANCE/mm²) AT 50ng DNA

R+   R-

80
70
60
50
40
30
20
10

SUBSTITUTE SHEET

Fig. 16.

Fig. 17.

Fig. 18.

Fig. 19.

Fig. 20.

PHORBOL ESTER INDUCED AND UNINDUCED CELLS

Fig. 21.

Fig. 22.

Fig. 23.

Fig. 24.

Fig. 25.

Fig. 26.

DNA IN PEG-PHASE(%CONTROL)

CONCENTRATION OF VP16 (M)

Fig. 27.

USING UV CROSSLINKING
TO DEMONSTRATE RETINOIC ACID INDUCED DNA PROTEIN
ASSOCIATION

PEG-PHASE DNA (%SHAM-TREATED CONTROL)

MINUTES
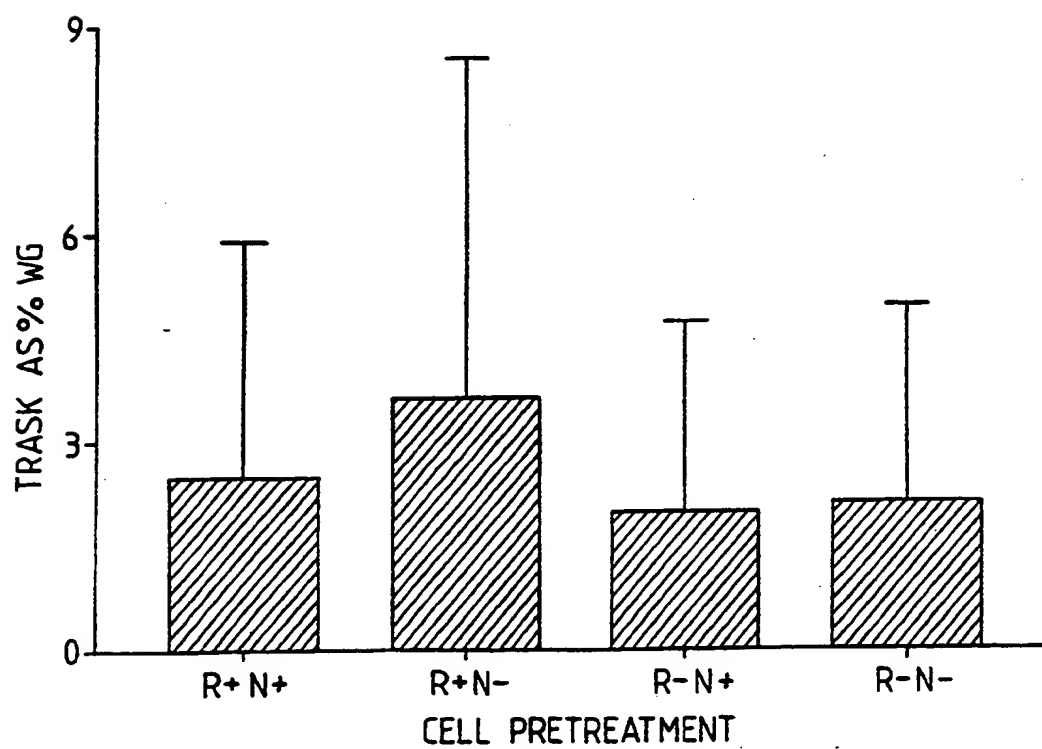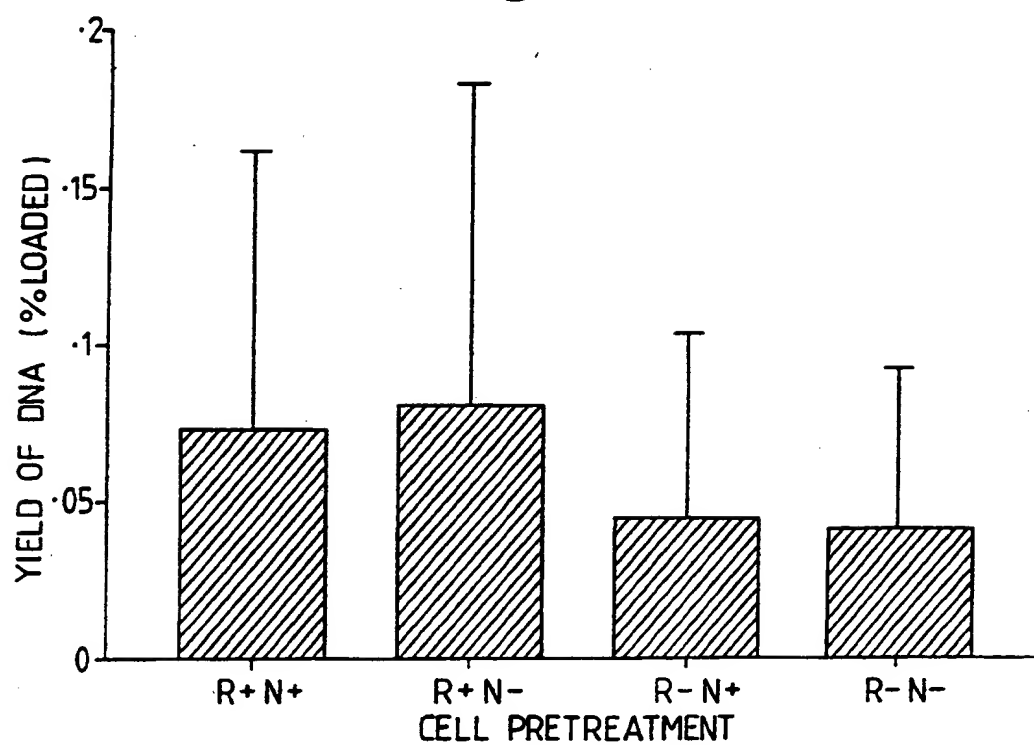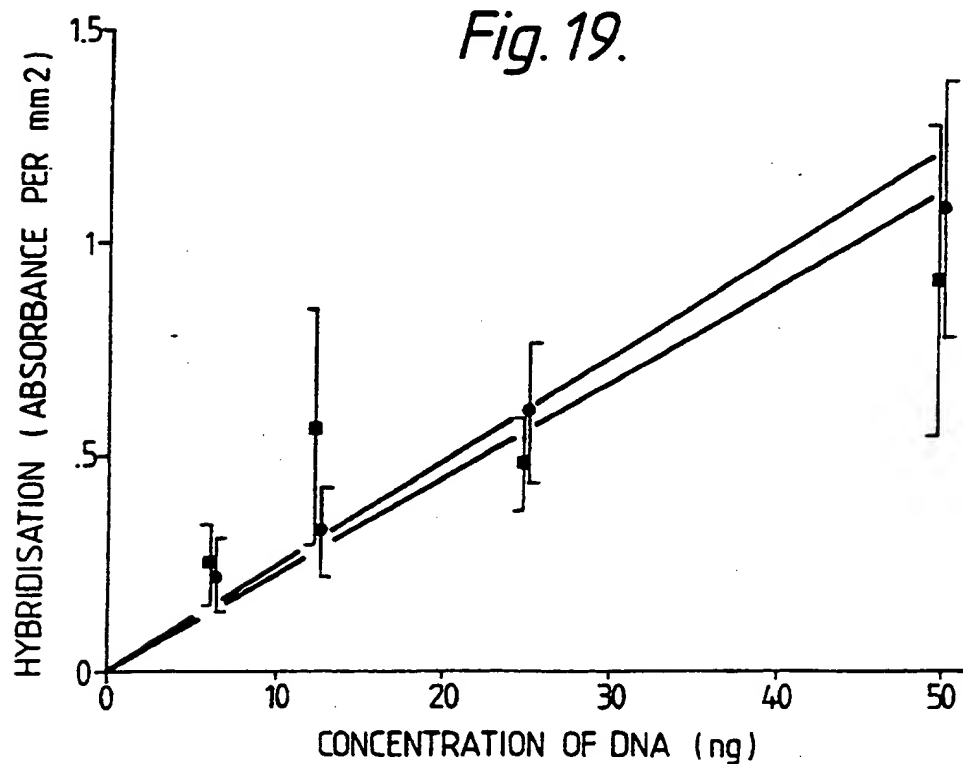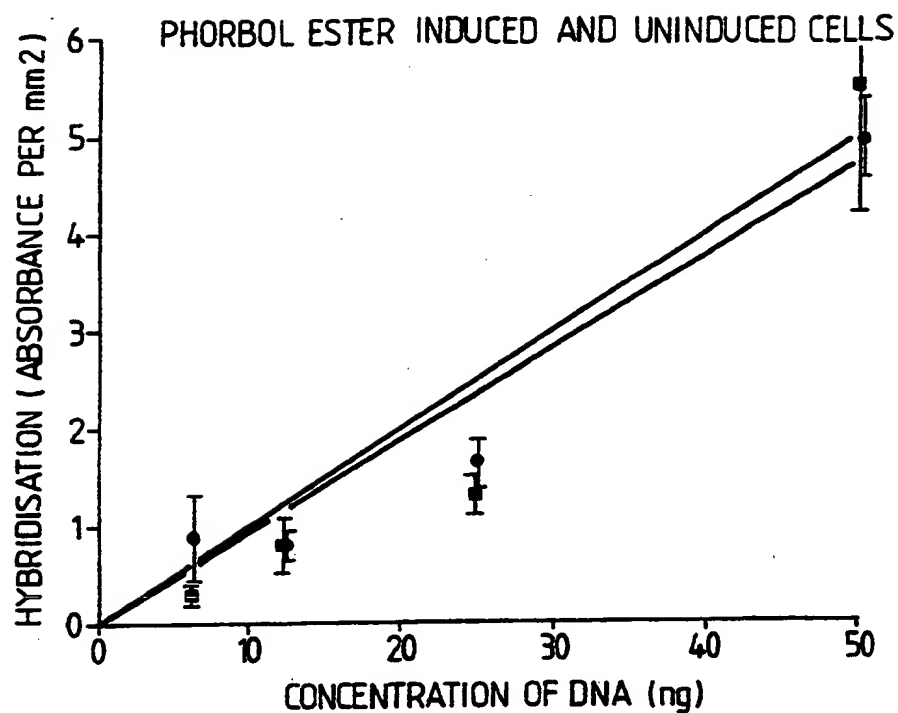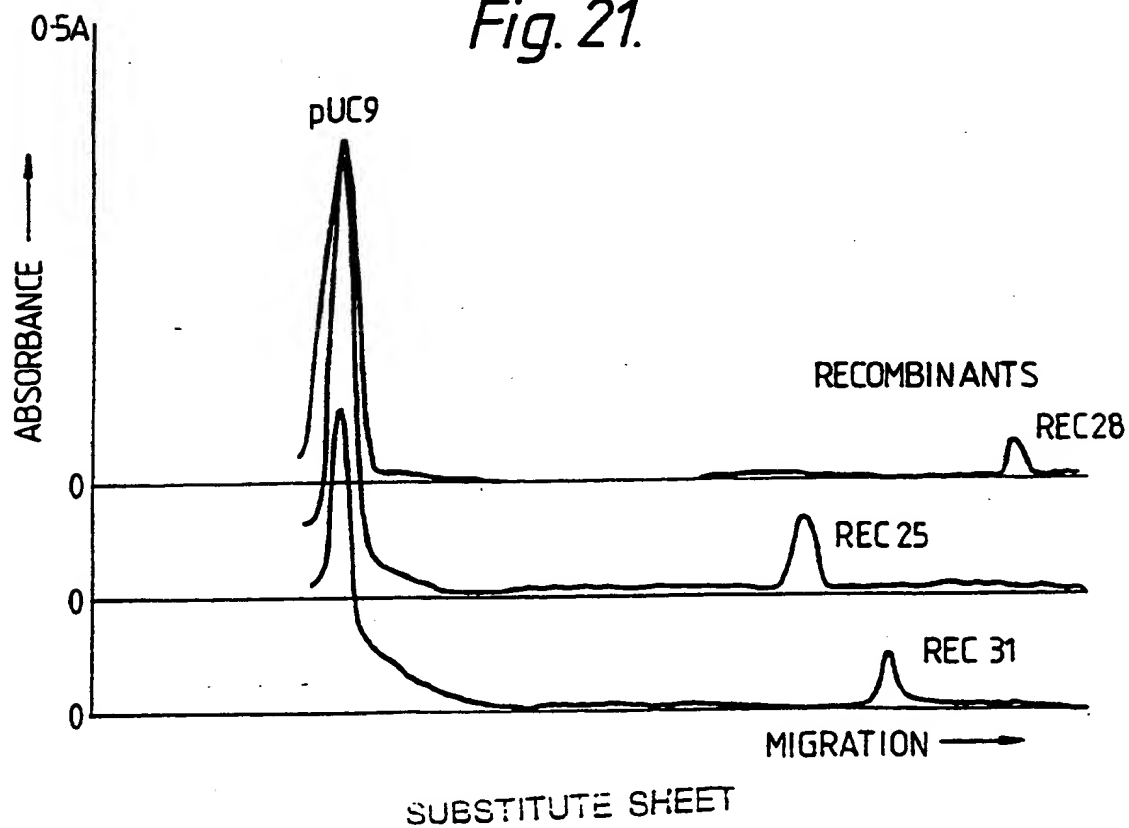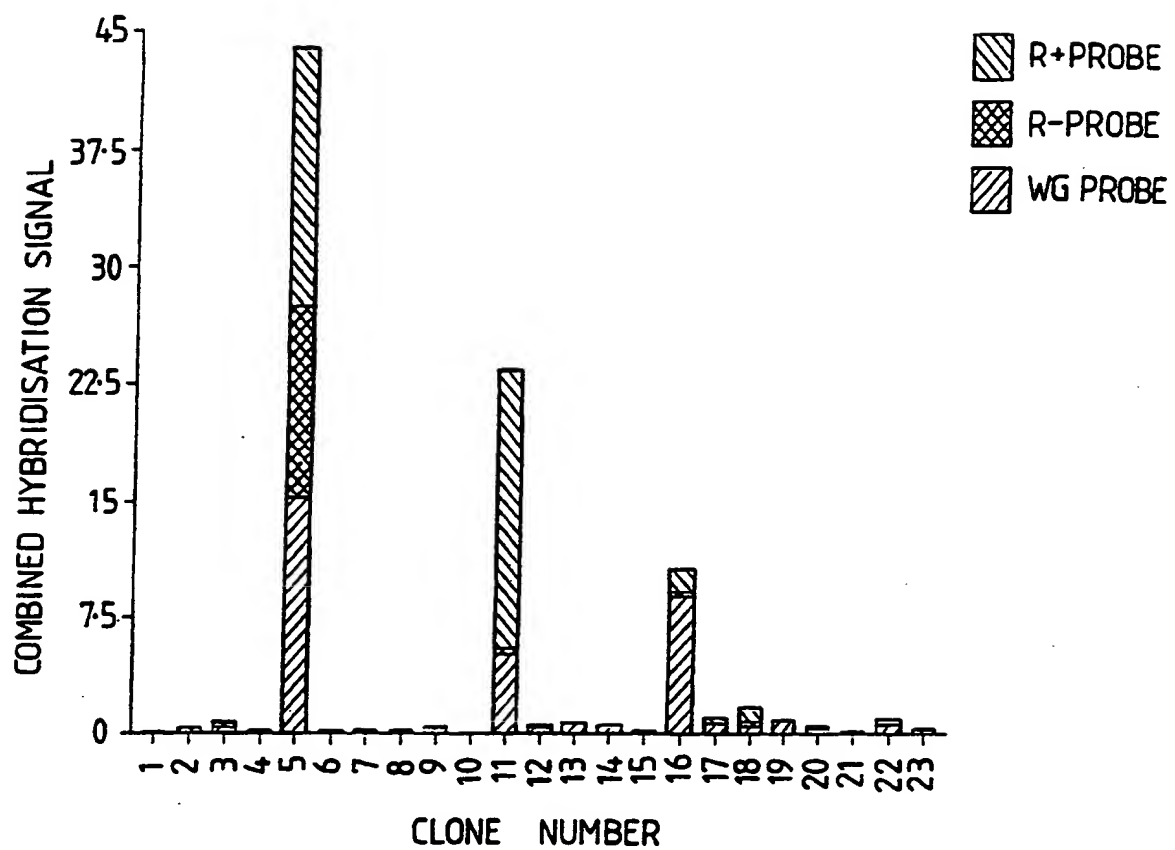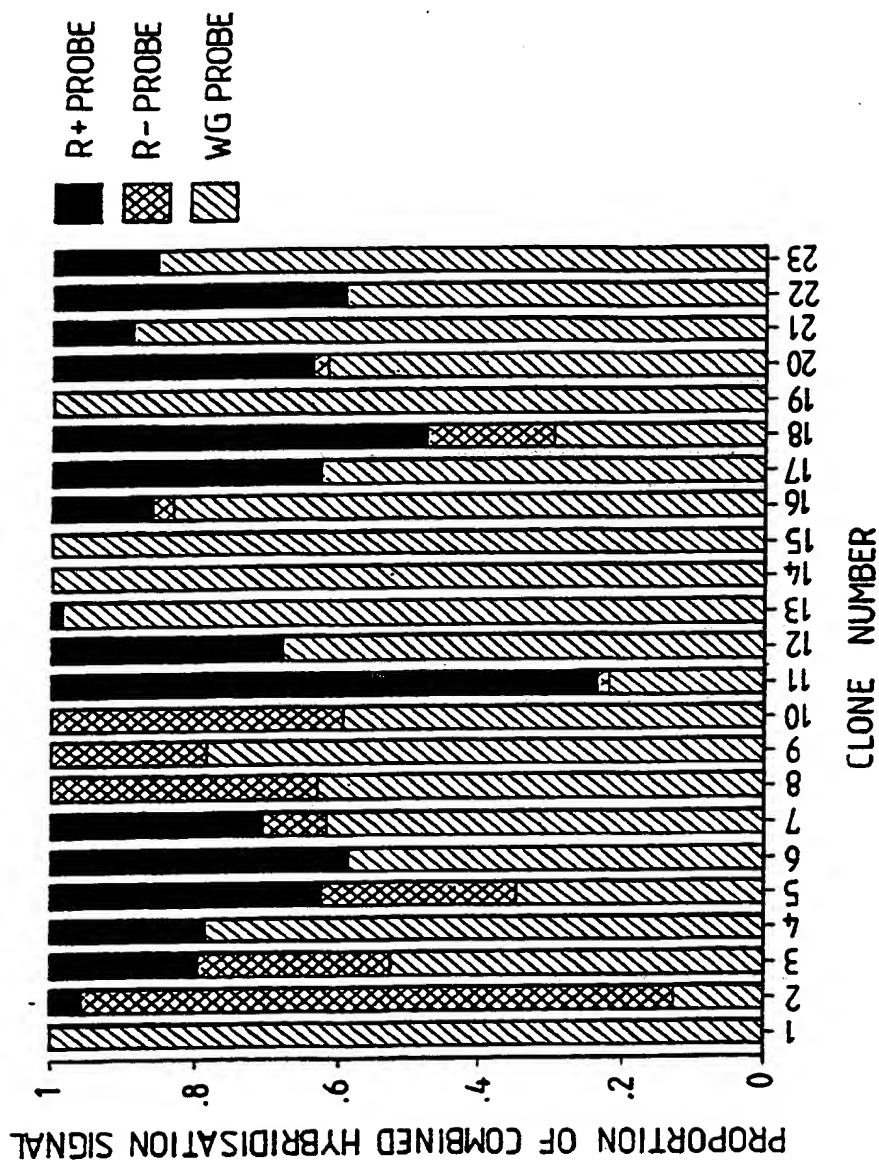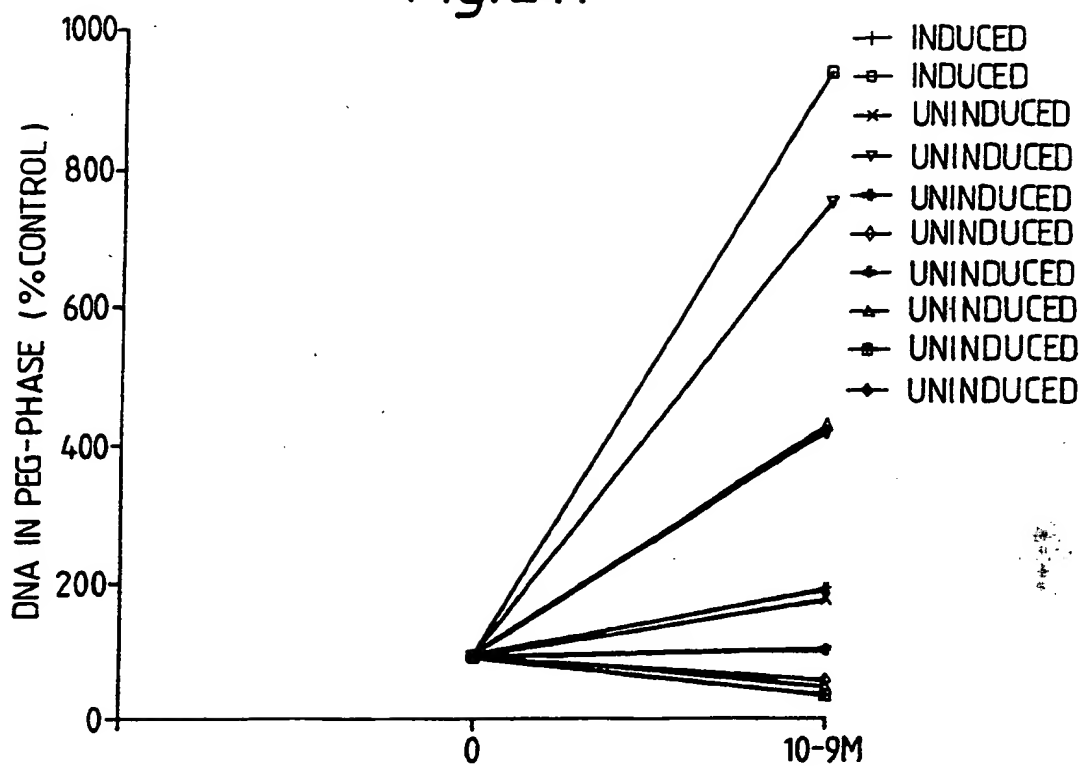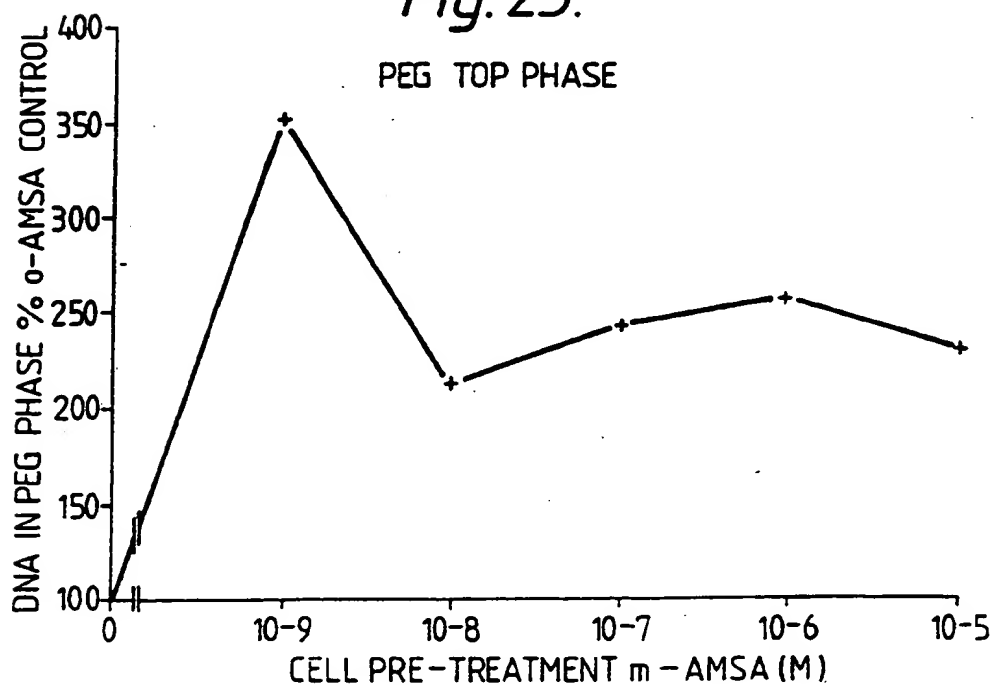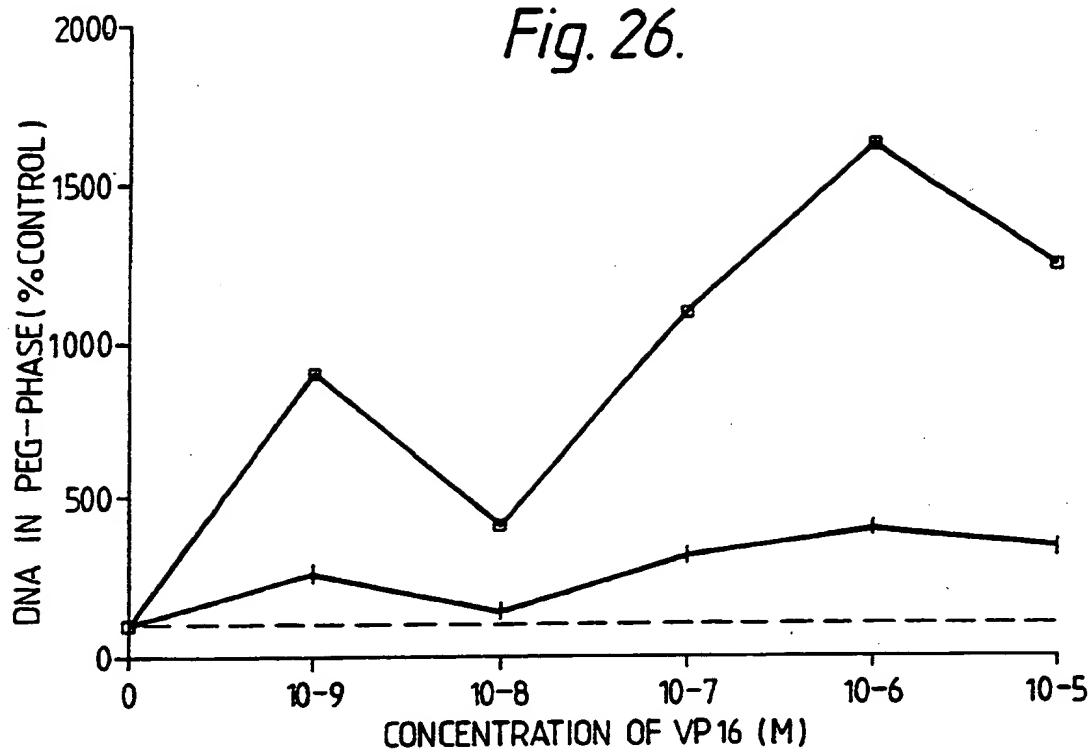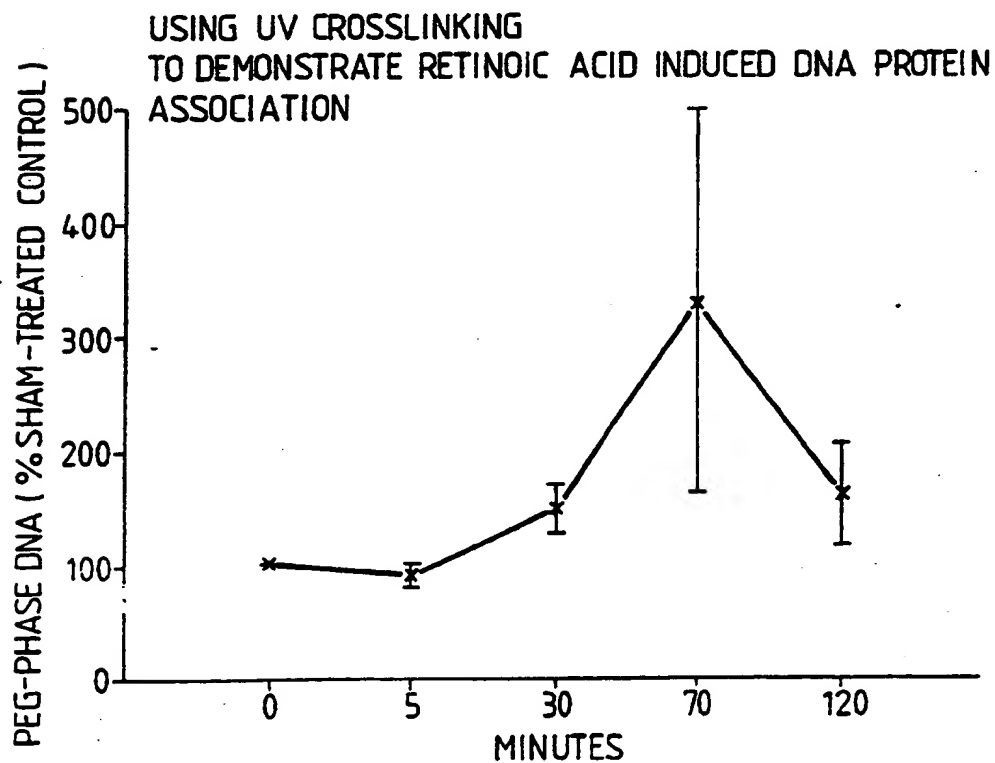
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) °

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC5	C 12 Q

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category °	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	The EMBO Journal, Vol. 3, No. 3, 1984, Douglas K. Trask et al.: "Rapid detection and isolation of covalent DNA/protein complexes: application to topoisomerase I and II.", see page 671 - page 676	1-12
A	Biochemistry, Vol. 20, 1981, Leonard A. Zwelling et al.: "Protein-associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4'-(9-acridinylamino)methanesulfon-m-anisidide and adriamycin", see page 6553 - page 6563	1-12
A	Chem. Pharm. Bull., Vol. 36, No. 8, 1988, Paul McGoff et al.: "Analysis of Polyethylene Glycol Modified Superoxide Dismutase by Chromatographic, Electrophoretic, Light Scattering, Chemical and Enzymatic Methods ", see page 3079 - page 3091	1

* Special categories of cited documents: ¹⁰

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th January 1990	3 1 JAN 1990

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	T.K. WILLIS